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THE DETERMINATION OF VITAMIN B_{12}-ACTIVITY IN THE ORGANS AND EXCRETA OF SHEEP

I. MICROBIOLOGICAL ASSAY METHODS

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(Accepted for publication 27th July, 1953.)

It seems probable from available evidence that vitamin B_{12} and the closely analogous vitamin B_{12a} (or B_{12b}) and vitamin B_{12c} all originate in nature only as products of the metabolic activity of certain micro-organisms. The chemical differences between these analogues are minor ones—the cyanogen group, which is attached rather loosely to the cobalt atom in the complex molecule of vitamin B_{12}, is replaced in vitamin B_{12a} (or B_{12b}) by a hydroxyl group, and in vitamin B_{12c} by a nitrite radicle; and there is little if any difference between their capacities to provide the nutritional requirements of higher animals and of exacting micro-organisms. These cobalamins (vitamin B_{12} and its analogues) are readily interconvertible—vitamins B_{12a} (B_{12b}) and B_{12c}, for example, are converted into vitamin B_{12} in dilute cyanide solution (Kaczka, Wolf, Kuehl and Folkers, 1950; Veer, Edelhausen, Wijmenga and Lens, 1950; Wijmenga, Veer and Lens, 1950; Lester Smith, 1950–1951; Boxer and Rickards, 1951; Jukes and Stokstad, 1951; Ungley, 1951–1952; Marston, 1952; Darken, 1953).

Other metabolic products of micro-organisms are known which share certain of the properties of the cobalamins but differ from them in important respects. These vitamin B_{12}-like substances occur along with vitamin B_{12} as products of bacterial activity in the contents of the rumen and of the lower levels of the alimentary canal, and in the faeces of animals and birds. Their existence was first indicated by the widely divergent activities exhibited by extracts of faeces, rumen contents, etc., towards the micro-organisms employed for the estimation of vitamin B_{12} (Coates, Ford, Harrison, Kon, Porter, Cuthbertson and Pegler, 1951).

Extracts of calf faeces prepared by boiling in dilute cyanide solution were found to contain four substances which exhibited vitamin B_{12}-activity for a mutant of *Escherichia coli*. One of these was vitamin B_{12} itself; the others were called fractions A, B and C. Fraction A was obtained in crystalline form and was provisionally designated cyano-ω-cobalamin. Vitamin B_{12} and fractions A, B and C differed markedly in their capacities to promote the multiplication of the test organisms *Euglena gracilis, Escherichia coli* and *Lactobacillus leichmannii*. Fractions A and B were inactive for the chick when given in the diet, but fraction A was active when injected, and when tested on one patient with pernicious anaemia its potency was little short of that of vitamin B_{12} (Coates, Ford, Harrison, Kon and Porter, 1952; Ford, Kon and Porter, 1952 a and b; Ford and Porter, 1952).

A crystalline substance (vitamin B_{12m}) isolated from the faeces of pigs by Wijmenga (1951) proved to be a cyanide complex with an absorption spectrum very like that of vitamin B_{12}. It promoted the growth of *L. leichmannii* but was less potent than vitamin B_{12}; it was clinically active in pernicious anaemia. A factor, probably the same as vitamin B_{12m}, was obtained from horse and cow manure.

Lewis, Tappan and Elvehjem (1952 a and b) reported the isolation of a crystalline cyanide complex, vitamin B_{12f}, from the faeces of rats. It promoted the growth of *L. leichmannii, E. coli* and *Euglena* but could not fulfil the vitamin B_{12} requirements of the rat or the chick.

Pfiffner, Dion and Calkins (1952) called the vitamin B_{12}-like factors isolated by them pseudo-vitamin B_{12}. From the harvest broth of a rumen anaerobe 3 pairs of pigments of the pseudo (Ψ) series were obtained, 3 cyano-cobalt compounds and 3 presumably hydroxo compounds. Two pairs of pigments which were obtained crystalline had a high vitamin B_{12}-activity for certain lactobacilli but could not replace the vitamin B_{12} necessary for chicks, rats and man. The third pair had no demonstrable biological activity. In the crystalline compounds the 5:6-dimethyl benziminazole group of vitamin B_{12} was replaced by adenine (Dion, Calkins and Pfiffner, 1952).

In more recent papers Kon and his co-workers have reported the results of an examination by ionophoresis of their own factors A, B and C and also pseudo-vitamin B_{12} and vitamin B_{12m} made available to them by Dr. Pfiffner and Dr. Wijmenga. A factor WR obtained from liver by Wijmenga was also examined. They found that even the crystalline substances, factor A, pseudo-vitamin B_{12} and vitamin B_{12m}, were not single entities but could be separated into several components. Thus factor A and vitamin B_{12m} contained the same major component with impurities which appeared to be vitamin B_{12}, pseudo-vitamin B_{12} and factor B. Microbiological assay also indicated the apparent identity of factor A and vitamin B_{12m} and served to differentiate these from pseudo-vitamin B_{12}. The activities of the different compounds determined by three methods of

assay were recorded. Taking the activity for *E. coli* (tube assay) as 100 in each case, the substances vitamin B_{12}, factor A, vitamin B_{12m}, pseudo-vitamin B_{12}, factor B and factor C showed relative activities of 100, 267, 282, 700, 770 and 8,500 (*E. coli*, plate assay) and 100, 83, 91, 293, 0·33 and 13·9 (*L. leichmannii*, tube assay) (Ford, 1953; Ford, Holdsworth, Kon and Porter, 1953; Holdsworth, 1953).

The experiments reported here were undertaken as a part of an investigation of the relationship between the cobalt content of the fodder and the amount of vitamin B_{12} that becomes available to the sheep. They concern mainly the procedures that were employed in the microbiological assays.

Preparation of Extracts for Assay.

Under a variety of experimental conditions cyanocobalamin proved to be more stable than its hydroxy analogue (Soars and Hendlin, 1951; Frost, Lapidus, Plaut, Scherfling and Fricke, 1952; Tarr, 1952). Cyanide has been reported to facilitate extraction of vitamin B_{12} from gut contents, faeces and sows' milk (Ford, 1952) and from eggs, tissues and faeces (Denton, Kellogg and Bird, 1952). This is probably because of the greater stability of cyanocobalamin during the extraction procedures. Incubation with cyanide liberated vitamin B_{12} from a compound in liver (Wijmenga *et al.*, 1950) and fish solubles (Hausmann and Mulli, 1952). In the presence of cyanide in the dark at pH 5·5 to 7 the vitamin B_{12} analogue[s] are converted to cyanocobalamin. Conversely, exposure to light at pH 5 or below causes a quantitative liberation of cyanide from its complex (Boxer and Rickards, 1951).

We have worked throughout in the presence of cyanide and have avoided exposure of solutions to bright light. When extracts were heated or brought to a reaction below pH 6 ruby glassware was used as far as possible. All samples which were not analysed within 24 hours of collection were stored at —20°C. Urine and faeces were collected from sheep confined in metabolism cages (Marston, 1935).

Urine.

Before storage, aliquots of the 24-hour samples of urine were treated with cyanide (0·5 ml. 1 p.c. KCN per 100 ml. urine), the reaction adjusted to pH 6·8 and the samples boiled for half a minute.

Immediately before assay the urine samples were thawed, diluted 10 times and autoclaved or steamed for 15 minutes after re-adjusting to pH 6·8. However, when this heat treatment was omitted the effect on the estimated vitamin B_{12} content appeared to be small: the data were insufficient for statistical treatment.

Faeces.

10 gm. aliquots of faeces were macerated in a Waring blendor in 200 ml. N/10 acetate buffer containing 10 mg. neutralized KCN. The mixture was transferred to a ruby flask in a final volume of 250 ml. and steamed for 30 minutes in a boiling autoclave at atmospheric pressure. After cooling, the extracts were centrifuged and filtered. Finally the reaction of the filtrates was brought to pH 6·8.

At first acetate buffers of pH 5·0 and 4·6 were used as extracting media. There was no significant difference between estimates of vitamin B_{12}-activity in samples of the same material extracted at pH 5·0 or 4·6 (Table 1). The small difference between the two estimated values was more often in favour of the pH 4·6 buffer, which was therefore used as the medium for extraction in all subsequent experiments.

TABLE 1.

Effect of reaction during steaming on the vitamin B_{12}-activity in sheep faeces.

Sample No. (Samples 1–7 not uniform)	Tube assay (*L. leichmannii*) μg. "B_{12}"/gm. wet weight pH 5·0	pH 4·6	Plate assay (*E. coli*) μg. "B_{12}"/gm. wet weight pH 5·0	pH 4·6
1 a and b*	0·27	0·27	0·55	0·58
2 „	0·35	0·44	0·55	0·63
3 „	0·28	0·28	0·65	0·71
4 „	0·25	0·25	0·40	0·43
5 „	0·30	0·29	0·43	0·39
6 „	0·80	0·75	3·8	3·6
7 „	0·43	0·51	3·3	4·2

* Sample a extracted at pH 5·0; sample b at pH 4·6.

This method of extraction proved the most satisfactory of a number that were tried. Digestion of faeces with an extract of pancreas[1], followed by 20 minutes' steaming, resulted in loss of activity, as did digestion with papain and takadiastase either before or after steaming. These results were obtained by plate assay with *E. coli;* if, however, the activity was estimated by *L. leichmannii,* there was only a very slight reduction when digestion followed steaming. The effect of digestion prior to steaming was not examined by this latter method.

TABLE 2.

Vitamin B_{12}-activity in liver A (L. leichmannii).

Sample No.	Assay No.	μg. "B_{12}"/gm. fresh liver	Treatment
L 24	59	1·37	Autoclaved 15 min. at 15 lb. (pH 4·6)
	61	1·34	
	Mean	1·355	
L 22	59	1·37	Autoclaved 30 min. at 15 lb. (pH 4·6)
	61	1·36	
	Mean	1·365	
L 23	59	1·32	Steamed 32 min. (pH 4·6)
	61	1·21	
	Mean	1·265	
L 21	59	1·60	Autoclaved 15 min. (pH 4·6) then digested 24 hours with pancreatic extract after adjusting to pH 6·9
	61	1·65	
	Mean	1·625	
L 1	59	1·37	Digested 24 hours, steamed 30 min. (pH 6·8 to 6·0)
	Overall mean	1·40	

Liver.

The maceration of the tissue in acetate buffer in the presence of cyanide was carried out in a manner identical with that used for faeces. After extraction the samples were cooled and filtered and the filtrates brought to pH 6·8. The extraction procedure was varied in order to determine the optimum method for liberating the vitamin B_{12}-activity. In Table 2 results have been presented for 5 different treatments of uniform samples of liver A.

[1] Prepared in the laboratory.

In a second experiment 12 uniform samples of liver B were assayed. In each case the macerated liver in acetate buffer at pH 4·6 was steamed for 30 minutes. Six of the samples were brought to neutrality and digested for 24 hours with pancreatic extract. Statistical analysis showed no significant difference between the vitamin B_{12} estimated by the two methods (Table 3).

TABLE 3.

Vitamin B_{12}-activity in liver B (L. leichmannii).

μg. "B_{12}"/gm. fresh liver		
Range	Mean	Treatment
0·54–0·62	0·57	Steamed 30 min.
0·52–0·61	0·57	Digested after steaming

In experiment 3, 12 uniform samples of Liver C were autoclaved for 15 minutes, 8 in pH 4·6 buffer and 4 in pH 5·6 buffer. Extra cyanide (5 mg. neutralized KCN) was added to the extracts of 4 of the 8 samples which had been heated in pH 4·6 buffer, after these were adjusted to pH 6·8. The 12 extracts were left overnight in the dark at room temperature before dilution for assay. Again there was no significant difference between the results obtained by the three methods (Table 4).

TABLE 4.

Vitamin B_{12}-activity in liver C (L. leichmannii).

Autoclaved 15 min. at pH 4·6		Autoclaved 15 min. at pH 5·6		Autoclaved 15 min. at pH 4·6 extra cyanide at pH 6·8	
Sample No.	μg. "B_{12}"/gm. fresh liver	Sample No.	μg. "B_{12}"/gm. fresh liver	Sample No.	μg. "B_{12}"/gm. fresh liver
1	0·64	7	0·68	2	0·61
3	0·66	8	0·64	4	0·68
5	0·67	9	0·62	6	0·72
12	0·64	10	0·68	11	0·64
Mean	0·65	Mean	0·65	Mean	0·66

TABLE 5.

Vitamin B_{12}-activity in caecum contents.

Sample No.	μg. "B_{12}"/gm. fresh weight Tube assay *(L. leichmannii)*	Plate Assay *(E. coli)*	Treatment
1	0·139	0·45	
2	0·135	0·47	
3	0·142	0·47	Autoclaved 15 minutes
4	0·140	0·48	
	Mean 0·139	Mean 0·47	
5	0·135	0·38	
6	0·130	0·35	
7	0·131	0·36	Autoclaved and digested
8	0·137	0·38	
	Mean 0·133	Mean 0·37	

Caecum contents.

A single test was carried out with caecum contents, using the same general procedure as for faeces and liver. On this occasion 8 uniform samples were assayed. After autoclaving for 15 minutes, half the samples were digested for 24 hours with pancreatic extract. Digestion caused a definite lowering of the vitamin B_{12} in caecum contents as estimated by the plate assay with *E. coli* (Table 5).

As a routine procedure we now macerate the tissue, faeces, rumen contents, etc., in an acetate buffer at pH 4·6 in dilute cyanide solution, autoclave for 15 minutes at 15 lb. pressure, cool, centrifuge and/or filter and adjust to pH 6·8 with KOH. The extract is then assayed for vitamin B_{12}-activity.

Recovery of added vitamin B_{12}.

The above procedure was further tested by adding pure vitamin B_{12} in known amounts to tissue samples immediately after maceration and before autoclaving. The total vitamin B_{12}-activity was determined in these samples and also in replicates to which no vitamin B_{12} had been added. The final assay was carried out by the tube method with *L. leichmannii* as the test organism. Results are presented in Table 6, A and B. (Methods of statistical treatment are given in Appendix II.)

TABLE 6A.

Recovery of pure vitamin B_{12} added to liver (L. leichmannii).

Assay No.	No. of replicate samples	B_{12} added per sample μg.	B_{12} found μg./10 gm. mean	5 p.c. fiducial limits for μg. B_{12}/10 gm.	Significance of deviation from quantitative recovery
76*	2 (10 gm.)	0	3·2	2·3–4·0	n.s.
	2 „	10	12·7	11·8–13·6	($P > ·05$)
88†	3 „	0	12·7	12·2–13·2	n.s.
	3 „	5	17·1	16·6–17·6	($P > ·05$)
92†	2 „	0	14·2	13·4–14·9	significant
	3 „	10	22·8	22·1–23·4	($P < ·05$)
93†	3 „	0	14·5	14·2–14·8	significant
	2 „	10	23·6	23·1–24·0	($P < ·05$)
94*	3 „	0	14·0	13·1–14·9	n.s.
	3 „	5	19·0	18·1–19·9	($P > ·05$)
	2 „	10	24·4	23·3–25·5	

TABLE 6B.

Recovery of pure vitamin B_{12} added to rumen contents (L. leichmannii).

Assay No.	No. of replicate samples	B_{12} added per sample μg.	B_{12} found μg./30 gm. /60 gm.	5 p.c. fiducial limits for μg. B_{12}/30 gm. /60 gm.	Significance of deviation from quantitative recovery
73*	3 (30 gm.)	0	7·4	5·6–9·1	n.s.
	3 „	6	14·0	12·2–15·7	($P > ·05$)
95*	3 (60 gm.)	0	0·79	0·49–1·09	n.s.
	3 „	1·5	2·23	1·99–2·47	($P > ·05$)
	3 „	3·0	3·53	3·29–3·77	

After extraction and adjustment to pH 6·8 extracts were left overnight: * in refrigerator; † at room temperature.

Different parts of the same liver were used in assays 88, 92 and 94, and the same extracts in assays 92 and 93.

MICROBIOLOGICAL ASSAY OF VITAMIN B_{12}-ACTIVITY IN THE EXTRACTS.

I. By the growth and lactic acid production of *Lactobacillus leichmannii* 313.

Technique.

The method used by us is a modification of methods previously described (Skeggs, Nepple, Valentik, Huff and Wright, 1950; Thompson, Dietrich and Elvehjem, 1950; Cooperman, Drucker and Tabenkin, 1951).

The organism is maintained by weekly subculture in a medium containing 10 p.c. dry skim milk, 0·5 p.c. yeast (Difco) and 0·25 p.c. hepamino (Evans), adjusted to pH 6·8 and autoclaved for 15 minutes at 15 lb.

The inoculum medium, in which the organism is grown for 18 hours prior to the assay, consists of the assay medium modified by the omission of ascorbic acid and the addition of 0·25 p.c. hepamino. The 18 hours culture in this medium is centrifuged, washed once with physiological saline and resuspended in saline. The opacity of the suspension, determined by visual comparison with Burroughs Wellcome opacity tubes, usually lies between 4 and 8. A further dilution of about 1 in 10 is made with saline. One drop of the final suspension is used to inoculate each assay tube.

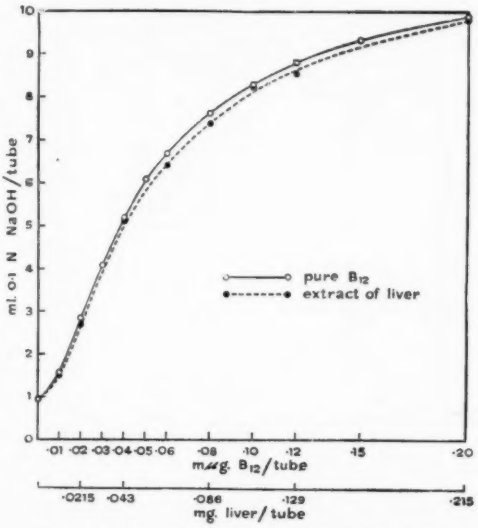

Fig. 1. Dose response curves for pure vitamin B_{12} and for an extract of liver *(L. leichmannii)*. (Assay 97.)

The assay is carried out in pyrex test tubes 16–17 mm. in internal diameter and 150 mm. in length. Each contains 5 ml. of assay medium and 5 ml. of test solution. From 6 to 12 levels of pure vitamin B_{12} have been used as standards, ranging from 0 to 0·2 mμg. B_{12} per tube. Extracts are assayed at 3 to 6 levels and 4 replicates of each level of standard and extract are employed. The tubes are plugged with cotton wool and autoclaved for 7 minutes at 15–16 lb. pressure. Before inoculation they are set out in 4 racks, one replicate in each, the positions of the tubes within the racks being randomized. They are incubated at 37° C. for 72 hours, after which time the lactic acid produced is titrated with N/10

NaOH. A dose response curve is drawn from the titrations of the standard tubes and the concentration of vitamin B_{12}-activity in the extracts is read from this (see Appendix II). In Fig. 1 are shown dose response curves for pure vitamin B_{12} (12 levels including the blank) and for an extract of liver (8 levels). A discussion of the forms of the dose response curves for pure vitamin B_{12} and for liver extracts is incorporated in Appendix II.

Assay medium.

TABLE 7.

Assay medium.

1	HCl-hydrolyzed casein	5 or 6 gm.
2	Glucose	20 gm.
3	Na acetate $3H_2O$	10 gm.
4	dl-tryptophane	100 mg.
5	l-cystine	100 mg.
6	Adenine SO_4	5 mg.
7	Guanine HCl	5 mg.
8	Xanthine	5 mg.
9	Uracil	5 mg.
10	Salts A	5 ml.
11	Salts C*	5 ml.
12	Pyridoxine HCl	2 mg.
13	Pyrodoxal HCl	2 mg.
14	Riboflavin	1 mg.
15	Nicotinic acid	1 mg.
16	Ca pantothenate	1 mg.
17	Thiamine HCl	1 mg.
18	Biotin	5 μg.
19	p-aminobenzoic acid	0·5 mg.
20	Folic acid	about 100 μg.
21	Tween 80	0·1 ml.
22	Malic acid	1 gm.
23	KCN	2·5 mg.
24	Ascorbic acid	500 mg.
	Adjust to pH 6·8 with KOH and dilute to	500 ml.

* Cooperman *et al.* (1951). NaCl was omitted because of its presence in the casein hydrolysate.

The first 19 constituents of the medium (Table 7), except No. 11, are present in the same concentration as in the medium of Skeggs *et al.* (1950). Certain factors affecting the choice of the remaining constituents are discussed below.

Folic acid. The dose response curves for pure vitamin B_{12} and for crude extracts (rumen contents and rat faeces) were found to be the same whether the medium contained 10 μg. or 100 μg. of folic acid per 500 ml. In an early experiment measuring the response to pure vitamin B_{12} in a medium without ascorbic acid, the lactic acid production was less when the medium contained 580 μg. folic acid per 500 ml. than with one-fifth or one-fifteenth of this amount.

Tween 80. The concentration of tween 80 in the assay medium is one tenth of that recommended by Skeggs *et al.* (1950) and Thompson *et al.* (1950).

When titrating the lactic acid produced by the assay organism we use bromthymol blue as an internal indicator and check the end point (pH 6·8) with a glass electrode. In the presence of tween 80, 1 ml. per 500 ml. medium, pH 6·8 was reached when the indicator was still yellow, while the blue-green colour of the ordinary end point did not develop until the pH was 7·5. With this amount of tween we found it necessary to use bromcresol purple as indicator. When the concentration of tween 80 was reduced to 0·1 ml. per 500 ml. medium, the colour change of the indicator occurred at almost the normal reaction, pH 6·8. The effect on the dose-response curve was small (Fig. 2): lactic acid production at the two lowest levels of vitamin B_{12} and in the blank was slightly reduced, while at the upper end of the curve it was increased. In view of these findings it seemed preferable to use the lower concentration of tween 80 in the medium.

Ascorbic acid and malic acid. Skeggs *et al.* (1950) recommended the inclusion of thiomalic acid in the medium as it fulfilled two functions:

(1) stimulation of the growth of the organism, which was apparently due to the organic acid, since malic and other related acids had a similar effect; and

(2) protection of vitamin B_{12} in crude extracts, which was also afforded by other reducing agents, e.g. ascorbic acid, thioglycollic acid, etc.

Since thiomalic acid was not available while this work was being carried out, malic acid was incorporated in the medium. Cooperman *et al.* (1951) reported that in the presence of cyanide it was not necessary to include a reducing agent. We found, however, that the response to vitamin B_{12} was considerably greater when ascorbic acid as well as cyanide was present in the medium. For this reason, we preferred to include it.

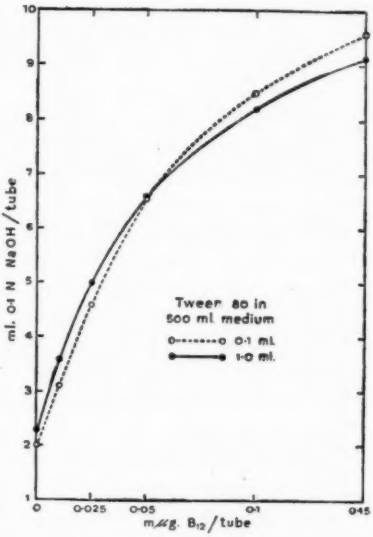

Fig. 2. The effect of the concentration of Tween 80 in the medium on the response to pure vitamin B_{12} *(L. leichmannii).*

Cyanide. The amount of KCN in the medium, 2·5 mg. per 500 ml., is certainly not toxic to the micro-organism. The response to pure vitamin B_{12} was the same at all levels whether the medium contained 0·25 or 2·5 mg. KCN per 500 ml., while with 25 mg. the response was very slightly greater.

Destruction of vitamin B_{12} by heating under alkaline conditions.

Thymidine and other purine and pyrimidine desoxyribosides promote the growth of the micro-organism in the absence of vitamin B_{12}. Their presence in tissue extracts will therefore interfere with the microbiological assay of the vitamin by this method. Heating the tissues in alkali destroys vitamin B_{12} and the residual activity is a measure of the desoxyribosides present (Hoffmann, Stokstad, Hutchings, Dornbush and Jukes, 1949; Lester Smith, 1950-51).

We have found that steaming for 30 minutes in N/10 NaOH destroys all the vitamin B_{12} in pure solution. In the presence of rumen contents, 16 p.c. wet weight, this procedure also appears to cause quantitative destruction, since less than 1 p.c. of the pure vitamin added to rumen contents was recovered. With liver, however, such treatment is insufficiently drastic. In two experiments 34 p.c. and 41 p.c. of added vitamin B_{12} were recovered. Of the amount estimated to be present in the livers at the beginning, 38 p.c. and 52 p.c. were recovered in the two experiments.

Autoclaving liver for 15 minutes at 15 lb. pressure in N/10 NaOH destroyed all but a trace of added vitamin B_{12}: about 2 p.c. remained and this small amount was believed to be within the experimental error of the method.

In kidney, brain and liver the alkali stable activity determined by alkaline autoclaving was between 2 and 6 p.c. of the total vitamin B_{12}-activity. It is evident that desoxyribosides do not interfere seriously with the estimation of vitamin B_{12} in these tissues.

Robinson, Williams and Brown (1952) reported that commercial liver extracts contained a factor which stimulated the growth of *L. leichmannii* and *E. coli* and which was not destroyed by boiling for 30 minutes at pH 10. It was not a desoxyriboside. Separation from vitamin B_{12} was effected by removing the latter with phenol. Östling and Nyberg (1953) also reported the presence in crude liver extracts of factors with vitamin B_{12}-activity which were not destroyed by boiling for an hour at pH 10—residual activity after such treatment varied from 0 to 87 p.c. in six liver extracts. The authors considered that two alkali stable factors for *L. leichmannii* were present in the extracts.

Our experience seems to show that fresh liver contains a factor which increases the resistance of vitamin B_{12} to destruction by alkali and that this protection is afforded to pure added vitamin B_{12} and to that present in liver in approximately equal measure. Frost, Fricke and Spruth (1952) found that destruction of vitamin B_{12} in liver extracts as determined by microbiological and rat assay "was not entirely complete even under conditions of drastic treatment with NaOH."

II. By the plate method of Harrison, Lees and Wood (1951).

In this method the concentration of active material in the extracts is measured by its capacity to promote the growth of a vitamin B_{12}-requiring mutant of *Escherichia coli*. The assay medium, containing agar and inoculated

with the test organism, is poured into petri dishes. After cooling, solutions of pure vitamin B_{12} and of the test substances are introduced into holes cut in the agar. The plates are incubated overnight and the diameters of the rings of growth are measured. The method has been described in detail by the authors. It is suitable for the assay of extracts containing not less than 0·005 μg. "B_{12}"/ml. It was not used for the assay of urine.

Dose response relationship.

It has been shown by Harrison *et al.* (1951) and confirmed by us that there is a straight line relationship between the diameter of the rings of exhibition and the logarithm of the dose of pure vitamin B_{12}, between the concentrations 0·005 μg./ml. and 0·2 μg./ml.

We found it convenient to use at least these two levels in every assay to give the standard dose response line. Test solutions were assayed at two or more concentrations likely to yield intermediate levels of vitamin B_{12}-activity. The question of choice of levels for standards and extracts is discussed in Appendix I.

The concentration of vitamin B_{12}-activity in test solutions was determined by comparing the diameters of the rings produced with those due to pure vitamin B_{12}. This was done either by reading from the standard line or by the use of a formula (see Appendix I).

For this method of estimation to be valid it is essential that the slope of the dose response line for the test solution should be the same as that of the standard line. Frequently, however, it was found that the slopes of the two lines differed significantly. This matter is further discussed below.

Faeces. The vitamin B_{12}-activity was liberated by steaming for 30 minutes in the presence of cyanide. The faecal extracts were usually assayed at two levels, one being five times more dilute than the other. Occasionally a ten times dilution was used.

An examination of 55 assays, each on 10 plates and comprising 32 faecal extracts, showed that in 49 cases the slope of the dose response line for the faecal extracts was greater than that of the standard line. Statistical analysis of individual assays showed that while this divergence from the slope of the standard line was in some instances not significant, in others it was significant ($P<·05$, $P<·01$ or $P<·001$).

In one test the assay medium was enriched by including the amino acids used in a tube assay with *E. coli* (Burkholder, 1951). We added 200 mg. each of glutamic acid, glycine, L-arginine, L-histidine, L-proline and DL-tryptophane to each 1,150 ml. of medium. Three levels of faecal extract, F, F/5, F/50, were tested on 20 plates. The response was linear but the slope of the line differed significantly from that of the three standards ($P<·001$). The slopes were:

Standards	5·33
Faecal extracts	6·12

Rumen contents. Extracts were prepared by autoclaving 40 gm. samples (wet weight) in 240 ml. pH 4·6 buffer in the presence of cyanide. They were assayed at two levels, R and R/5 (Table 8).

TABLE 8.

Vitamin B_{12}-activity in rumen contents, plate assay with E. coli.

Sheep No.	μg. "B_{12}"/gm. dry wt. reading 1/5 level from standard line (mean)	Significance of difference between slopes of lines for extracts and standards* (individual sets of 8–10 plates)	
1075 (1 sample)	1·35	n.s.†	6 sets
		$P < ·05$	2 sets
		$P < ·01$	1 set
W3 (1 sample)	0·99	n.s.†	3 sets
9134 (3 samples)	0·98	$P < ·001$	6 sets
7049 (3 samples)	6·1	n.s.†	6 sets

* When the slopes of the lines differed significantly that of the extracts was greater than that of the standards. † $P > ·05$.

TABLE 9.

Vitamin B_{12}-activity in the intestinal contents of sheep—No. 1130 (E. coli).

Level of Intestinal Tract	Replicate sample No.	μg. "B_{12}"/gm. dry wt. reading 1/5 level from standard line. Mean of 10 plates	Slope: Standard line	Slope: Unknown	Significance of difference between slopes
Small Intestine	1	1·09	4·96	3·50	$P < ·001$
	2	1·00	4·96	3·82	$P < ·001$
	Mean	1·045			
Caecum	1	3·6	5·24	5·22	n.s.†
	2	3·7	5·49	5·58	n.s.†
		3·8	5·31	5·44	n.s.†
	3	3·8	5·56	5·72	n.s.†
		3·6	5·40	5·58	n.s.†
	4	3·9	5·43	5·29	n.s.†
		3·7	5·03	4·85	n.s.†
	Mean	3·7			
Colon	1	3·1	4·46	5·01	$P < ·001$
	2	2·8	4·46	4·72	n.s.†
	Mean	2·95			
Rectum	1	3·3	4·65	5·58	$P < ·001$
	2	3·5	4·65	5·44	$P < ·001$
	Mean	3·4			

† $P > ·05$.

A fourth sample from sheep No. 7049 (see Table 8) was assayed at 4 levels between 0·2 and 0·005 μg. "B_{12}"/ml. In this case the response was not linear over the whole range. The results were as follows:

Mean slopes (30 plates)

Standards	5·38
R, R/5	5·23
R/5, R/10, R/20	6·47

The slope of the line through the last 3 levels was significantly greater than that of the standards ($P < ·001$).

Other intestinal contents. As with the rumen contents extracts were prepared by autoclaving in the presence of cyanide, and were assayed at two levels, 1 and 1/5. Specimens of the contents of the small intestine, caecum, colon and rectum, obtained from one sheep, were examined. The caecum contents were assayed on 70 plates, the other specimens on a total of 50 plates. The results of the assays are presented in Table 9.

TABLE 10.

Vitamin B_{12}-activity in liver A (E. coli).

Sample No.	Plates	μg. "B_{12}"/gm. fresh liver reading 1/5 level from standard line	Slope: Standard line	Slope: Unknown	Significance of difference between slopes	Treatment
L 24	1–10	1·53	5·34	4·61	$P < ·001$	Autoclaved
	51–60	1·28	5·12	4·65	$P < ·01$	15 min.
	61–70	1·31	4·96	4·86	$P > ·05$	at 15 lb.
	71–80	1·24	5·07	4·56	$P < ·05$	
	Mean	1·34	*5·12	4·67	$P < ·01$	
L 22	11–20	1·28	5·31	4·86	$P < ·01$	Autoclaved
	51–60	1·22	5·12	4·94	$P > ·05$	30 min. at 15 lb.
	Mean	1·25				
L 23	21–30	1·28	5·09	4·86	$P > ·05$	32 min.
	61–70	1·31	4·96	4·65	$P < ·05$	Steamed
	Mean	1·295				
L 21	81–90	1·31	5·21	3·93	$P < ·001$	Autoclaved and digested
L 1	31–40	1·49	5·37	3·15	$P < ·001$	Digested
	71–80	1·26	5·07	3·31	$P < ·001$	and
	81–90	1·45	5·21	3·22	$P < ·001$	autoclaved
	Mean	1·40				
Overall Mean		1·32				

* When compiling results from several sets of plates variability in responses between sets was considered as part of the error. We now consider it necessary to use at least 3 sets of 10 plates for each extract.

Liver. Liver samples previously assayed by the tube method with *L. leichmannii* (Tables 2 and 3) were also examined by the plate method using *E. coli* as the test organism (Tables 10 and 11).

TABLE 11.

Vitamin B_{12}-activity in liver B (E. coli).

Sample No.	μg. "B_{12}"/gm. fresh liver reading 1/5 level from standard line. Mean of 10 plates	Slope: Standard line	Slope: Unknown	Significance of difference between slopes	Treatment
4	0·56	5·38	4·93	$P < ·05$	
12	0·65	5·37	5·15	$P > ·05$	Steamed
8	0·67	5·41	4·85	$P < ·01$	30
3	0·62	4·96	4·65	$P > ·05$	minutes
5	0·57	5·34	5·15	$P > ·05$	
Mean	0·61				
6	0·59	5·38	4·05	$P < ·001$	
7	0·63	5·37	3·51	$P < ·001$	Digested
11	0·63	5·41	3·82	$P < ·001$	after
1	0·61	4·96	2·79	$P < ·001$	steaming
2	0·59	5·34	3·58	$P < ·001$	
9	0·67	5·40	3·72	$P < ·001$	
Mean	0·62				

Following digestion of liver there was a marked fall in the slope of the dose response line (Tables 10 and 11). As a corollary there was a wide difference between the estimates of vitamin B_{12} obtained by reading from the standard line at the two assay levels:

	μg. "B_{12}"/gm. Level 1/5	Level 1
Table 10 L 21	1·31	0·86
L 1	1·40	0·77
Table 11 Mean of 6 samples	0·62	0·34

DISCUSSION.

It is apparent from the experimental results presented that substances with vitamin B_{12}-activity are as effectively liberated from their compounds in the liver, faeces, and gut contents of sheep by heat treatment alone as by pancreatic digestion after heating. Since the latter process was found to have adverse effects in the plate assay, its use was abandoned.

Some consideration was given to the optimum reaction at which heating should be carried out. A slightly acid medium might be expected to be more effective in liberating vitamin B_{12} but it might also lead to a loss of the cyan group from its complex. However, under the conditions used by us, there was nothing to choose between extracting in dilute cyanide solution at pH 4·6, 5·0

or 5·6 or extracting at pH 4·6 and adding extra cyanide after adjusting to pH 6·8.

The extraction procedure which was finally adopted was further tested by adding pure vitamin B_{12} to tissues before autoclaving, and determining the amount recovered. This was also a test of the validity of the tube assay with *L. leichmannii* by means of which the activity in the extracts was measured. Results are presented in Tables 6 A and B. It would seem that there was no significant loss of added vitamin except in one instance (assays 92 and 93) when the extracts were left overnight at room temperature instead of in a refrigerator. There was insufficient evidence to decide whether this loss was caused by the higher temperature of overnight storage.

In the plate assay with *E. coli* mutant as the test organism we have repeatedly observed a lack of parallelism between the dose response lines for the standards and the test solutions. For faecal extracts the slope of the line was usually greater than that of the standard line and sometimes markedly so. For rumen contents a similar divergence in slope was at times observed and in one instance it was found that while the response line between levels R and R/5 was parallel with the standard line, at lower levels it had a much greater slope ($P<\cdot001$).

The explanation for these facts may lie in the presence in certain extracts of some substance not adequately supplied by the assay medium. Enriching the medium with amino acids, however, did not prevent a highly significant difference in the slope of the two lines when faecal extracts were assayed (v.s.).

It seems more likely that the phenomenon is due to the multiplicity of vitamin B_{12}-active substances present in the extracts. Ford (1953) has reported that in the plate assay with *E. coli* the response to pseudo-vitamin B_{12} was "markedly non-linear."

In only one instance did an extract of gut contents give a dose response line of slope less than that of the standard line. This was a specimen of small intestine contents. The difference in the slopes of the lines was highly significant ($P<\cdot001$).

The fall in the slope of the dose response line following digestion of liver seems to point to the production of a substance which inhibited the growth of the micro-organism when the extract was assayed in its original concentration, i.e. 4 p.c. liver. When the extract was diluted 5-fold the inhibition was no longer apparent and the digested and undigested extracts gave the same estimate for vitamin B_{12} (Tables 10 and 11). Moreover, a comparison of Tables 10 and 11 with Tables 2 and 3 shows a close approximation in the vitamin B_{12} estimated in liver by the tube method with *L. leichmannii* and the plate method (calculated only from the 1/5 level).

The interference with the assay caused by digestion was not due to liberation of methionine, although this was apparent. The addition of methionine to

pure vitamin B_{12}, ratio 10,000 to 1, had a negligible effect on the diameter of the rings although a hazy growth occurred around them.

It is probable that the inhibiting substance produced from liver by digestion was also present in smaller concentration in liver subjected to heat treatment, since the slope of the dose response line for heated liver extracts was consistently less than that of the standard line, although the difference was frequently not statistically significant.

Ford (1953) pointed out that the factor WR isolated from liver by Wijmenga contained a substance which inhibited the growth of *E. coli*.

Enzymic digestion following heat treatment of liver was without effect in the tube assay with *L. leichmannii*.

It was thought until recently that whatever method was used to estimate vitamin B_{12} the same results should be obtained and that any differences might be ascribed to experimental error. It is now known that materials rich in micro-organisms may contain several different vitamin B_{12}-like substances, at least five having been partially described. As these differ in their activity for different organisms, the results of an assay will, in certain materials, depend on the method by which the assay is carried out.

We found that extracts of sheep faeces always showed greater vitamin B_{12}-activity for *E. coli* than for *L. leichmannii*, the difference being 5-fold and 8-fold in two animals receiving cobalt by mouth (Dawbarn, Hine and Hughes, 1952). Similar discrepancies are apparent in assays of vitamin B_{12} in the contents of rumen, caecum, colon and rectum (Table 12).

TABLE 12.

Vitamin B_{12}-activity in the gut contents of sheep.

Sheep No.	Section of gut	μg. "B_{12}"/gm. dry weight *E. coli* C	*L. leichmannii* L	Ratio C/L
W 3	Rumen	0·96	0·40	2·4/1
1075	"	1·44	0·54	2·7/1
9134	"	0·98	0·34	2·9/1
7049	"	6·00	1·26	4·8/1
1130	Caecum	3·7	1·11	3·3/1
"	Colon	2·95	1·13	2·6/1
"	Rectum	3·4	1·12	3·0/1

SUMMARY.

Materials with vitamin B_{12}-activity were extracted from the faeces, gut contents and tissues of sheep and the total activity in the extracts was measured by a tube assay using *L. leichmannii* 313 as the test organism and by a plate assay with an *E. coli* mutant.

Extractions were carried out in ruby glassware by heating the tissues and other test substances in N/10 acetate buffers in dilute cyanide solution. There was no significant difference in the estimated vitamin B_{12} when the pH of the buffers was 4·6, 5·0 or 5·6, or when extraction at pH 4·6 was followed by the addition of extra cyanide after adjusting to pH 6·8. Digestion with pancreatic extract after heating was without effect in the tube assay *(L. leichmannii)* but interfered with the plate assay *(E. coli)*. Recovery tests were carried out.

The tube assay method was based on published methods in current use. Modifications introduced by us have been described.

Steaming for 30 minutes in N/10 NaOH completely destroyed vitamin B_{12} added to rumen contents but not that added to liver. Consequently to differentiate between desoxyribosides and vitamin B_{12} in liver, alkaline autoclaving was used.

In assays of faecal extracts or gut contents by the plate method the slope of the dose response line was frequently significantly greater than that of the standard line.

With liver extracts, on the other hand, the slope of the line was slightly less than that of the standards and this divergence of slope was greatly accentuated by pancreatic digestion.

For faecal extracts and gut contents the plate assay with *E. coli* gave a considerably higher estimate of vitamin B_{12}-activity than the tube assay with *L. leichmannii*. For liver the two methods gave the same result within the experimental error.

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APPENDIX I.

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APPENDIX I. STATISTICAL ANALYSIS OF DATA.

PLATE ASSAY USING *E. COLI.*

Calculation of results.

In the present series of assays, the standards used have been

S_1 — 0·2 μg. B_{12}/ml.
S_2 — 0·005 μg. B_{12}/ml.

i.e., the concentrations are in the ratio 1:40. The extracts, however, have usually been assayed at the two levels, 1, 1/5. The standards were chosen as above to cover the entire range where a linear response was known to hold, so that for the many cases where results were obtained by reading from the graph of the standard line, these would be the best estimates obtainable by this method. The graphical method, however, is wasteful of information, ignoring as it does

(i) the necessity of testing the parallelism of the two response lines, as the assay is valid only where they are parallel;

(ii) half the information on the slope of the response curve, since only the slope of the standard line enters the calculation of the results.

Where results are calculated by the method of maximum likelihood, using the formula given below, it is preferable that the same ratio of dilutions for both standard and unknown should hold. The formula is given in general terms, to cover both equal and unequal dilution ratios.

We use the following notation:

	Mean diameter of ring	log. (conc. B_{12})
Standards	S_1, S_2	x_{s1}, x_{s2} resp.
Extract (1)	U_1, U_2	x_{u1}, x_{u2} resp.
Extract (2)	V_1, V_2	x_{v1}, x_{v2} resp.

Let $x_{s1} - x_{s2} = \log$ (ratio of concentrations of standards) $= d$

$x_{u1} - x_{u2} = x_{v1} - x_{v2} = \log$ (ratio of concentrations of extracts) $= \lambda d$

We may calculate the slope of the response curve for the standards as

$$m_s = \frac{S_1 - S_2}{d}$$

or for the extracts as

$$m_u = \frac{U_1 - U_2}{\lambda d}$$

The significance of the difference of the slopes of standards and extracts may then be tested by taking

$$t = \frac{\lambda(S_1 - S_2) - (U_1 - U_2)}{\sqrt{\frac{2s^2}{r}(\lambda^2 + 1)}}$$

where s^2 is the estimated variance of a single observation, having n degrees of freedom;

r the number of plates comprising the assay;

t Student's t-variate with n degrees of freedom (tabled in Fisher and Yates' "Statistical Tables").

If the slope of neither extract differs from the slope of the standards, we may calculate a maximum likelihood estimate of the slope of the common response curve by weighting the separate estimates inversely as their variances, thus obtaining

$$m = \frac{S_1 - S_2 + \lambda(U_1 - U_2 + V_1 - V_2)}{d(1 + 2\lambda^2)} \quad . \quad . \quad . \quad . \quad . \quad \text{(i)}$$

If the slope for one unknown differs significantly from that of the standards, the maximum likelihood estimate (for calculating the concentration in the second unknown) becomes

$$m = \frac{S_1 - S_2 + \lambda(V_1 - V_2)}{d(1 + \lambda^2)} \quad . \quad . \quad . \quad . \quad . \quad \text{(ii)}$$

Let

$$R_U = \tfrac{1}{2}(U_1 + U_2 - S_1 - S_2)$$

Then, provided the slope of the first unknown and that of the standards do not differ,

$$x_{U1} - x_{S1} = \frac{R_U}{m} + (\lambda - 1)\frac{d}{2}$$

and hence

concentration B_{12} in first unknown = antilog $\frac{R_U}{m} + (\lambda - 1)\frac{d}{2} + x_{S1}$ where m is estimated by (i) or (ii) above, whichever is appropriate.

If the slope of the unknown differs significantly from that of the standards, no valid estimate of the concentration of B_{12} in the extract can be made (Wood, 1944; Finney, 1944; Gridgeman, 1944; Jerne and Wood, 1949).

Fiducial limits.

The p% fiducial limits of x (log. concentration of extract) are

$$x + \frac{g}{1-g}(x - \bar{x}) \pm \frac{t}{m(1-g)}\sqrt{A(1-g) + B(x - \bar{x})^2}$$

where $A = \frac{s^2}{2r}$

$$B = \frac{2s^2}{rd^2(1 + 2\lambda^2)}$$

$$g = \frac{t^2 B}{m^2} \quad t = \text{Student's } t\text{-variate at } (100 - p) \text{ p.c. level on d.f. of } s^2.$$

$$\bar{x} = \tfrac{1}{2}(x_{S1} + x_{S2})$$

and m is calculated from formula (i) above (Irwin, 1943; Fieller, 1944). Where m is

calculated from the standards and one extract only, fiducial limits are given by the same expression, but in this instance

$$B = \frac{2s^2}{d^2(1 + \lambda^2)}$$

The use of range in estimating s^2.

Where, as in this instance, many assays are being performed, considerable labour is necessary to compute s^2 by the method of least squares; e.g., for a 10-plate assay, an analysis of a randomized block type is done giving a subdivision of degrees of freedom as follows:

Variation due to	Degrees of freedom
Plates	9
Solutions	5
Error	45
Total	59

The randomized block analysis by range, as outlined by Hartley (1950), has been tried, and proved eminently suitable for this work. Once an estimate of s^2 has been obtained by this method, it may be used in t-tests, taking the degrees of freedom given in Hartley's table. In the example above, the loss of efficiency is slight, s^2 as estimated by range having 41 degrees of freedom, while the least squares estimate has 45.

APPENDIX II. STATISTICAL ANALYSIS OF DATA.

TUBE ASSAY USING *L. LEICHMANNII*.

Standard curve.

An attempt was made to find the form of the standard curve between values of dose of ·01 and ·15 mμg. B_{12} per tube. In the assays considered, a cubic was found to give a satisfactory fit in 93, 94, 95, 97, 98 and 99, but not in 88, 90 and 92. Where cubics fitted, they were found to have the same shape (i.e. their partial regression coefficients did not differ from one another by more than would be expected by chance).

For the purpose of finding the concentration of solutions of vitamin B_{12}, it was decided to read back from a freehand graph, since any personal bias in drawing the graph or reading back from it was less than the deviations of the cubic from the observed points. These deviations did not seem to be random, and hence would appear as biases at particular levels of the dose.

Curve for liver extracts.

To determine whether the shape of the dose-response curve for liver extracts was the same as that of the standard curve, assays 97, 98 and 99 were performed, 97 with 8 levels of dilution, and 98 and 99 with 7 (Fig. 1).

In assays 97 and 98, the vitamin B_{12} content of extracts calculated from different levels did not differ significantly, but in 99, one level gave a figure significantly lower than the others, $P < ·01$. The results were taken to indicate that in these assays the shape of the curve for liver extracts was the same as that for pure vitamin B_{12}. However, such uniform results were not always obtained. Data from three assays in which significant differences between levels were found are presented in Table 13. They indicate the magnitude of the differences which may occur between the estimates of vitamin B_{12} content calculated from different levels of assay.

TABLE 13.

Assay	Level of concentration of liver extract	μg. "B_{12}"/gm. fresh liver. Mean for 3 samples	Significance of difference between levels
88	a	1·22	$P < \cdot05$
	1·25 × a	1·23	
	2 × a	1·32	
	2·5 × a	1·29	
	5 × a	1·28	
	Mean	1·27	
93	b	1·56	$P < \cdot01$
	1·6 × b	1·55	
	2 × b	1·46	
	3·2 × b	1·35	
	4 × b	1·43	
	6·4 × b	1·34	
	Mean	1·45	
94	c	1·42	$P < \cdot05$
	1·25 × c	1·41	
	2·5 × c	1·41	
	4 × c	1·35	
	Mean	1·40	

Recovery of added vitamin B_{12}.

Analyses of variance were performed on figures calculated as μg. B_{12} per 10 gm. of samples minus the theoretical amount added.

In assay 94, for example, eight samples were taken. To three of these no vitamin B_{12} was added, to another three 5 μg. B_{12}, and to the remaining two 10 μg. B_{12} was added. The eight samples were assayed at four different levels corresponding approximately to concentrations of vitamin B_{12} of ·035, ·045, ·07, ·11 mμg. per tube. The form of the analysis was as follows:

Variation due to	Degrees of freedom
Amount of B_{12} added (B)	2
Error (1)	5
	7
Levels	3
Levels × B	6
Error (2)	15
	31

For the three sets of samples (no added B_{12}, 5 μg. added, 10μg. added), means of the amount of vitamin B_{12} per 10 gm. were found, and the 5 p.c. fiducial limits for these means were calculated using Error (1).

In assay 90 but in none of the others, the analysis of variance revealed an interaction ($P < \cdot001$) between levels and amount of vitamin B_{12} added. The results of this assay were therefore discarded.

THE GROWTH OF POLIOMYELITIS VIRUS IN TISSUE CULTURE

I. THE USE OF A STATIONARY BOTTLE TECHNIQUE

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(From the Commonwealth Serum Laboratories, Melbourne).

(Accepted for publication 18th September, 1953.)

Enders, Weller and Robbins (Enders *et al.*, 1949; Weller *et al.*, 1949) have reported the growth of poliomyelitis virus in cultures of human embryonic non-nervous tissue and the same authors (Robbins *et al.*, 1950) found that growth was accompanied by specific cytological changes in the tissue culture. Other American workers (Smith *et al.*, 1951; Syverton *et al.*, 1951; Youngner *et al.*, 1952a, 1952b, 1952c; Ledinko *et al.*, 1952; Riordan *et al.*, 1952) soon confirmed these observations and showed that certain adult tissues as well as monkey testicular tissue would provide suitable tissue explants. All such studies of cytological changes were carried out using a roller tube technique.

Experiments with poliomyelitis virus in tissue culture were initiated at the Commonwealth Serum Laboratories about two years ago. After initial difficulties had been overcome it became apparent that stationary tissue cultures would yield viral infective titres as high as those obtained in roller tube cultures. Melnick and Riordan (1952) have reached similar conclusions recently. It is the purpose of this paper to describe in detail the stationary bottle technique now in use at these Laboratories.

MATERIAL AND METHODS.

Preparation of glassware and rubber stoppers.

These are brought to the boil in 0·1 p.c. NaOH solution and then rinsed thoroughly in three changes of hot distilled water. After draining, the glassware is placed in metal canisters or stoppered with gauze-wrapped cotton plugs and dry heat sterilized at 150° C. for 1 hour. The rubber stoppers are autoclaved at 20 lb. pressure for 20 minutes, in suitable test tubes plugged as above.

Culture medium components.

(a) Hanks' Balanced Salt Solution (B.S.S.) is prepared according to the formula of Dr. J. H. Hanks, as given by Weller and Enders (1948).

	Gm./litre		
NaCl	8·0	$CaCl_2$	0·14
KCl	0·4	Na_2HPO_4	0·06
$MgSO_4.7H_2O$	0·1	KH_2PO_4	0·06
$MgCl_2.6H_2O$	0·1	Glucose	1·0

5 ml. of 0·4 p.c. aqueous phenol-red solution is added for each litre and the B.S.S. is sterilized by autoclaving at 10 lb. pressure for 10 minutes. An autoclaved 1·4 p.c. solution of $NaHCO_3$ is prepared separately and aliquots of this solution are added to the B.S.S. just prior to use. Glass distilled water is used throughout.

(b) *Chick embryo extract (CEM).* This is prepared from 9- to 11-day-old embryos, using the whole embryo including head and eyes. After harvesting aseptically into a Waring blendor an equal volume of B.S.S. (containing 1 ml. of the 1·4 p.c. $NaHCO_3$ solution to each 120 ml. of B.S.S., 0·1 mg. streptomycin and 100 units penicillin per ml.) is added and the whole mixed for three minutes. After standing at room temperature for 30 minutes the suspension is centrifuged at 2,500 r.p.m. for 30 minutes. The resulting supernatant, representing an extract of a 50 p.c. suspension of chick embryo tissue and designated CEM_{50} is rapidly frozen in an alcohol-dry ice bath and stored at —20° C. Before use it is thawed and clarified by centrifugation at 3,000 r.p.m. for 30 minutes.

(c) *Chicken plasma.* Using a sterile technique, roosters are bled by heart puncture into 20 ml. syringes containing 0·05 mg. of heparin in sterile solution in 1 ml. of 0·85 p.c. NaCl solution (sufficient to prevent 20 ml. of blood clotting). The plasma, obtained by centrifugation, is immediately freeze dried in 2 ml. quantities and stored under vacuum at 4° C. to be reconstituted with sterile distilled water as required.

(d) *Ox serum ultrafiltrate.* An adaptation of the method given by Wilson and Holiday (1933) is used to prepare ox serum ultrafiltrate which gives no precipitate when tested with 20 p.c. trichloracetic acid. After the addition of stock 0·4 p.c. phenol-red solution to give a final concentration of 0·002 p.c. it is Seitz filtered and the pH adjusted to approximately 7·2 by aseptically introducing 5 p.c. CO_2. The ultrafiltrate is stored under 5 p.c. CO_2 in tightly stoppered bottles at 4° C.[1]

(e) *Horse serum.* The serum from four horses, each of which has been tested individually regarding suitability for tissue culture work, is pooled, filtered through a D_6 Seitz filter pad and stored in sterile tightly stoppered Pyrex bottles. No difference could be detected between inactivated (heated at 56° C. for 30 minutes) and raw serum in tissue culture experiments but the former is used in all media requiring serum. Serum stored for periods up to twelve months at 4° C., after inactivation, has proved satisfactory in use.

Complete culture media.

After testing various media we adopted the procedure recommended by Youngner *et al.* (1952b) and use two complete media (Medium A and Medium C). All components which are used have previously been tested for support of fibroblastic outgrowth and poliomyelitis virus growth.

(a) Medium A is used for production of the fibroblastic outgrowth from tissue explants, prior to virus inoculation. This medium comprises 20 p.c. CEM_{50}, 40 p.c. horse serum, 40 p.c. B.S.S. containing 1 ml. of 1·4 p.c. $NaHCO_3$ solution per 40 ml. of B.S.S., and penicillin and streptomycin to a final concentration of 50 units and 0·05 mg. per ml. respectively.

(b) Medium C is used to replace medium A after virus inoculation and for any subsequent medium changes. This medium comprises 10 p.c. CEM_{50}, 10 p.c. horse serum, 20 p.c. ox serum ultrafiltrate, 60 p.c. B.S.S., and $NaHCO_3$ and antibiotics as for Medium A.

Tissue for explants.

Under ether anaesthesia, testes are removed aseptically from healthy rhesus monkeys. The testes are carefully dissected free of the tunica albuginea and as much as possible of the mediastinum testis is removed. The tissue is minced with scissors in a sterile petri dish

[1] Ox serum ultrafiltrate suitable for tissue culture is obtainable from the Commonwealth Serum Laboratories, Melbourne.

and stored in B.S.S. (containing 1 ml. of 1·4 p.c. $NaHCO_3$ solution to each 120 ml. of B.S.S. and penicillin and streptomycin to a final concentration of 100 units and 0·1 mg. per ml. respectively) at 4° C. The tissue is used the day it is collected, as poor results have been obtained with tissue stored for longer periods.

Preparation of the tissue culture.

One drop of rehydrated chicken plasma is spread with a suitably bent pipette in a band 1 cm. wide along the inner surface of a 20 ml. volume bottle. The bottle in which Commonwealth Serum Laboratories penicillin is dispensed is suitable. Four pieces of testis, cut on a pyrex glass plate with a No. 11 Bard Parker blade, each approximately 1 mm. in diameter, are introduced into the bottle and arranged in a straight line along the centre of the plasma band. Two drops of chick embryo extract (CEM_{50}) diluted 1:3 in B.S.S. are added, the bottle gently rocked to induce clotting, which is allowed to occur with the bottle lying horizontally in a rack. After coagulation, 1 ml. of Medium A is added to each bottle, a rubber stopper inserted and the bottles incubated at 36° C. in a horizontal position with the tissue submerged. Satisfactory fibroblastic outgrowth is obtained in six days and no necessity to change the medium during this period has been found.

For the purpose of poliomyelitis virus isolation from faecal extracts, tissue cultures are prepared as before except that eight to twelve pieces of tissue, minced finely with scissors instead of being cut by a scalpel, are introduced into each bottle and 2 ml. of Medium A added. This medium is replaced by 2 ml. of fresh Medium A on the third day after the tissue cultures are prepared.

Inoculation of the tissue culture.

Before using for attempted virus isolation or growth all tissue cultures are examined by means of a 32-fold magnification plate microscope and those showing unsatisfactory fibroblastic outgrowth discarded. After the removal of the fluid medium the cultures are inoculated with 0·1 ml. of faecal extract, virus suspension or virus suspension-antiserum mixture as required, followed 10 minutes later by the addition of 1 ml. of Medium C.

A detailed description of the methods used is given in another paper (Bazeley and Thayer, 1954). In the work reported here the following poliomyelitis virus type strains were used to inoculate the tissue cultures: Mahoney Type 1 (Brunhilde), MEF 1 Type 2 (Lansing), and Saukett Type 3 (Leon).[2]

These three strains were used because of their ability to produce definite cytological changes in the fibroblastic outgrowth (Youngner *et al.*, 1952b). We have found these changes occur in four days or less.

RESULTS.

Cytopathogenic effect of poliomyelitis virus on the fibroblastic outgrowth.

Evidence of the growth of a poliomyelitis virus is shown by the change in the appearance of the fibroblasts four to twelve days after the addition of the faecal extract or virus suspension, such change being inhibited by a specific poliomyelitis type antiserum. Examined microscopically at 50-fold magnification the presence, in the periphery of the outgrowth zone, of cells containing larger and coarser cytoplasmic granules than found in the surrounding cells of normal appearance is the earliest sign of virus induced degeneration. An impres-

[2] These three poliomyelitis virus type strains were kindly supplied by Dr. Jonas Salk, Virus Research Laboratory, University of Pittsburgh, Pittsburgh, Pennsylvania, U.S.A.

sion of diffuse granularity follows as the patches of cells in which degeneration occurs spread and eventually coalesce. The granular changes are followed by various degrees of cytolysis and eventual loss of the normal highly refractile appearance of the nucleus. The zone of the fibroblastic outgrowth ceases to enlarge and as cytolysis continues generally diminishes in size.

Poliomyelitis virus titre in tissue culture.

The titre of poliomyelitis virus in tissue culture is taken as the highest dilution of a standard volume of virus suspension which when inoculated into tissue culture produces a definite cytopathogenic effect on the fibroblastic outgrowth in eight days.

Using the stationary bottle technique as described in this and another paper (Bazeley and Thayer, 1954), the three stock poliomyelitis virus type strains, Mahoney, MEF 1 and Saukett, have been passaged through fifteen successive cultures and tissue culture titres for each strain of between 10^{-2} and 10^{-3} were regularly obtained. These titres are comparable with those reported (Youngner *et al.*, 1952b; Ledinko *et al.*, 1952; Melnick *et al.*, 1952) for various poliomyelitis virus strains grown in roller tube tissue culture.

Monkey testicular tissue fibroblastic outgrowth variation.

A marked variation between monkeys in the ability of testicular tissue explants to produce a satisfactory fibroblastic outgrowth has been found. Rigidly controlled attention to the collection of the testicular tissue, preparation of the explants and the tissue culture medium has not produced satisfactory outgrowth from the second testis where the first has given poor results. The percentage of tissue cultures with satisfactory fibroblastic outgrowth from a series of seven monkeys is given in Table 1.

TABLE 1.

A variation in the fibroblastic outgrowth obtained from tissue cultures of individual rhesus monkey testicles.

Monkey	Percentage of tissue cultures with satisfactory fibroblastic outgrowth	
	Testicle 1	Testicle 2
A	40	46
B	70	77
C	89	91
D	95	96
E	36	65
F	91	91
G	98	96

SUMMARY.

A stationary bottle technique for the growth of poliomyelitis viruses in tissue culture has been developed and described. Tissue culture titres of the poliomyelitis viruses comparable with those reported by other workers using roller tube cultures have been consistently obtained.

In tissue culture a variation in the fibroblastic outgrowth from testicular tissue obtained from a number of rhesus monkeys is noted.

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THE GROWTH OF POLIOMYELITIS VIRUS IN TISSUE CULTURE

II. ISOLATION AND TYPING OF POLIOMYELITIS VIRUS STRAINS USING A STATIONARY BOTTLE TECHNIQUE

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In a previous paper (Bazeley and Thayer, 1954) a stationary bottle technique for obtaining fibroblastic outgrowth from monkey testicular tissue and satisfactory growth of poliomyelitis virus therein was described. In this paper it is proposed to give in detail the methods being used at these Laboratories for the isolation and typing of poliomyelitis virus.

METHODS.

Preparation of faecal extracts.

A 20 p.c. suspension of the faecal sample in sterile distilled water is prepared and centrifuged at 3,500 r.p.m. for 30 minutes. To the decanted supernatant is added penicillin and streptomycin to a final concentration of 1,000 units and 5·0 mg. per ml. respectively (Banker and Melnick, 1951). It is then centrifuged at 18,000 r.p.m. for 30 minutes. The resulting supernatant is distributed in 2 ml. quantities into rubber stoppered vials, rapidly frozen in an alcohol-dry ice bath and stored at —78° C. in a dry ice cabinet until required. Poliomyelitis viruses stored in this manner have been still viable after eighteen months.

Isolation of poliomyelitis virus from faecal extracts.

Faecal extracts are inoculated into duplicate minced tissue cultures as Youngner *et al.* (1952a) have shown that these greatly overcome the difficulty of toxic faecal material. Inoculation is made into 6- or 7-day-old tissue cultures showing satisfactory fibroblastic outgrowth, the fluid medium is removed and 0·1 ml. of faecal extract introduced. This is allowed to remain in contact with the tissue for approximately 10 minutes after which 2 ml. of Medium C (Bazeley and Thayer, 1954) is added and the cultures reincubated at 36° C. After four days the fluid medium is removed, discarded and replaced with 2 ml. of fresh Medium C. On the eighth day after inoculation the supernatant of both bottles is removed, pooled and 1 ml. quantities in rubber stoppered vials are rapidly frozen and stored at —78° C. All cultures from faecal extracts are harvested and stored in this manner, irrespective of the presence or not of fibroblastic degeneration, in order that the supernatants may be examined in tissue culture bottles containing four tissue explants.

Preparation of specific poliomyelitis virus type antisera.

The Mahoney Type 1 (Brunhilde), MEF 1 Type 2 (Lansing) and Saukett Type 3 (Leon) poliomyelitis antisera were prepared by us in rhesus monkeys, at the Walter and Eliza Hall Institute for Medical Research, Melbourne, using these virus strains. They were grown

in monkey testicular tissue cultures and given as an intramuscular injection of 1 ml. of culture supernatant, containing live poliomyelitis virus, mixed with an equal volume of an adjuvant (Salk *et al.*, 1951). Two such intramuscular injections were given at an interval of 14 days and two weeks later the monkeys were bled aseptically by heart puncture. The poliomyelitis antisera thus prepared were quite type specific with a tissue culture titre of 10^{-2} to 10^{-3} against specific poliomyelitis virus having a tissue culture titre of 10^{-2} and were used diluted 1:5 in inactivated horse serum throughout the work reported.

Typing of poliomyelitis virus in tissue culture.

A series of eight suitable bottles, each containing four tissue explants, after growing for six or seven days to obtain satisfactory fibroblastic outgrowth, is used for the typing of each sample. Duplicate bottles are used both for the control, without antisera, and for aliquots of the sample mixed with each of the three poliomyelitis type antisera.

0·15 ml. of the sample, eight-day supernatant harvested from a minced tissue-faecal extract culture, or other supernatant, is mixed with an equal volume of each of the three type antisera diluted 1:5 as described earlier, giving a final antiserum dilution of 1:10. The mixture is allowed to stand at room temperature for 1 hour before inoculating into the tissue culture. The fluid medium is removed from the bottles and 0·1 ml. of the supernatant or of the supernatant-antiserum mixture introduced. After this has been in contact with the tissue for approximately ten minutes 1 ml. of Medium C is added. The bottles are then reincubated and examined under 50-fold magnification every second day for signs of degeneration in the fibroblastic outgrowth. Early degeneration indicates proliferation of virus and lack of degeneration indicates neutralization of the virus by specific antiserum. We have found that poliomyelitis virus induced fibroblastic degeneration generally occurs by the eighth day after inoculation and is quite distinct. Beyond twelve days fibroblastic degeneration begins in uninoculated control tissue cultures and the line of demarcation between control and virus affected tissue culture is difficult to determine. Where further investigation is required, the supernatants from the inoculated control tissue cultures are harvested and stored as previously described.

Titration of poliomyelitis virus suspensions in tissue culture.

Serial tenfold dilutions of the poliomyelitis virus suspension to be titrated are made in balanced salt solution (Weller and Enders, 1948) containing 1 ml. of the stock 1·4 p.c. $NaHCO_3$ solution for each 120 ml. of B.S.S. 0·1 ml. of each dilution is inoculated as described previously into a duplicate set of bottles each containing four tissue explants.

The bottles are examined microscopically on the eighth day after inoculation and the tissue culture titre is given as the highest dilution producing definite fibroblastic degeneration in that time.

RESULTS.

Two series of faecal samples have been examined by the methods described. The first series consisted of thirty-four specimens collected during 1951 from poliomyelitis patients admitted to the Infectious Diseases Hospital, Fairfield, Melbourne. The second consisted of eighty-four faecal samples collected in the period August, 1952, until May, 1953, from paralytic and non-paralytic poliomyelitis patients at Fairfield, various Melbourne, Victorian and Tasmanian (five specimens) hospitals. Where possible, the country and interstate specimens were forwarded to these Laboratories packed in dry ice. The number of poliomyelitis virus strains isolated and typed in these two series is shown in Table 1.

TABLE 1.

A summary of results obtained in the isolation and typing of poliomyelitis viruses from faecal extracts, using tissue cultures in a stationary bottle technique.

Year	No. of faecal specimens examined	No. of each type isolated				Isolation rate
		Type 1	Type 2	Type 3	Mixed Type 1 and 3	
1951	34	15	0	2	1	53 p.c.
1952-53	84	0	8	2	0	12 p.c.

These figures show that there was a change in the type of poliomyelitis virus prevalent during the two periods and that the isolation rate (approximately 12 p.c. in the second series when Type 2 virus strains were prevalent) has fallen short of the isolation rate, 53 p.c., in the first series when the majority of the strains isolated were Type 1. These results are in keeping with the observations reported by Robbins *et al.* (1950) and Youngner *et al.* (1952b) that there is a wide variation in the capacity of various poliomyelitis virus strains, particularly those of Type 2 and Type 3, to induce cytologic changes. The figures shown for the second series therefore may not be a true representation of the actual number of virus strains present as some may be non-cytopathogenic under the conditions used for isolation and typing. We are now investigating the use of various other tissues and techniques in an attempt to improve the isolation rate.

Along with the change in prevalent type, it is of interest to note the presence of Type 3 viruses in the two series.

SUMMARY.

The isolation of poliomyelitis virus strains from faecal specimens and their subsequent typing using specific poliomyelitis antisera, produced by the immunization of rhesus monkeys with live poliomyelitis virus, is described. As shown by tissue culture technique, a change in the type of poliomyelitis virus prevalent in Victoria during the year 1951 and that during the period, late 1952 and early 1953, is reported.

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THE EFFECT OF BODY WEIGHT, AND AGE AT OVARIECTOMY, ON THE RESPONSE OF MICE TO OESTRONE

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In a series of experiments recently undertaken in this laboratory, large colonies of 500 or more ovariectomized mice have been required for studies on the vaginal response to oestrogens. It has not been possible to accumulate this number of mice in the short space of time which has hitherto been thought desirable for the production of a homogeneous colony. If in the collection of the animals successive batches give a different mean response the final colony will be more variable, the accuracy of any tests done with it will be reduced, and the advantages of using the large colony may be lost. Two possible causes of such heterogeneity may be the effect of body weight, and the age at which ovariectomy is carried out. In this paper these problems are investigated with particular reference to the intravaginal route of administration, although in the case of the effect of body weight observations for the subcutaneous route are also included.

MATERIALS AND METHODS.

Preparation of colonies.

Three colonies, to be called I, II and III of 571, 195 and 305 albino mice respectively, have been used in this work. Colonies I and II were prepared in the standard manner described by Biggers (1951), the required number of mice being accumulated, and then ovariectomized and primed together. They were subsequently used in tests at fortnightly intervals. Colony III was prepared in the manner shown in Table 1. Approximately 30 weanling female mice (21 days old) were selected each week for 10 weeks at random from the breeding colony and divided into classes A, B and C. Thus ten groups of mice in three classes, i.e. 30 sub-groups, were collected in all. Three weeks after collection, each class A and B was ovariectomized, and following this all of class A were regularly primed at fortnightly intervals by the subcutaneous administration of 1 μg. of oestrone in nut oil. Two injections of 0·05 ml. were given 24 hours apart. The mice of sub-group C were all ovariectomized three weeks after the last group was collected. All animals were primed simultaneously one week later, the mice then being ready for the tests. The mice of class C underwent the standard procedure (Biggers, 1951).

Biological assay.

Oestrone was administered in two injections in 24 hours, nut oil being used as solvent for subcutaneous administration and distilled water for intravaginal administration. The prepara-

tion of solutions, and the taking and reading of vaginal smears, followed the procedures described by Emmens (1950a) and Biggers (1951).

TABLE 1.

Dates of collection of Colony III and the subsequent treatment of its classes.

A. Dates of collection.

Group No.	Date of collection	Group No.	Date of collection
1	31/7/52	6	4/9/52
2	7/8/52	7	11/9/52
3	14/8/52	8	18/9/52
4	21/7/52	9	25/9/52
5	28/7/52	10	2/10/52

B. Dates of ovariectomy and priming.

Date	Subgroups ovariectomized	Subgroups primed
25/8/52	1A, 1B	—
1/9/52	2A, 2B	1A
8/9/52	3A, 3B	2A
15/9/52	4A, 4B	1A, 3A
22/9/52	5A, 5B	2A, 4A
29/9/52	6A, 6B	1A, 3A, 5A
6/10/52	7A, 7B	2A, 4A, 6A
13/10/52	8A, 8B	1A, 3A, 5A, 7A
20/10/52	9A, 9B	2A, 4A, 6A, 8A
27/10/52	10A, 10B, 1–10C	1A, 3A, 5A, 7A, 9A
10/11/52		1–10A, 1–10B, 1–10C

C. Dates of tests and weighing.

17/11/52	Test I
31/11/52	Test II
7/12/52	Weighed
14/12/52	Test III

RESULTS.

Body weight.

Effect of body weight on response. The results to be presented in this section have been obtained during the course of another investigation using Colonies I and II (Biggers, Claringbold and Emmens, 1954), one being used for subcutaneous tests and the other for intravaginal tests. The response of each animal to a median effective dose (M.E.D.) of oestrone was determined on two occasions, 14 days apart, the animals being weighed at a stage during this interval. Table 2 contains the results of these tests for both colonies. All animals were weighed to the nearest 0·5 gm., and since the animals used in the work on the subcutaneous method were more variable (as they happened to be older) than those used in the work on the intravaginal method, the former have been grouped to the nearest gram. In each test the regression of the probit of response on body

weight was determined using the methods described by Tocher (1949), and the Tables given by Finney (1952). If the smallest animals are the most sensitive to a given dose of the hormone this regression coefficient will have a negative sign. Table 3 contains the estimates of the regression coefficients and their standard errors for the data in Table 2. Since the regression coefficients are not independent estimates, a weighted regression cannot be simply obtained. Even if independence is assumed, and the regression coefficients are combined inversely to their variances, the weighted mean value does not differ significantly from zero. For this reason the analysis has not been pursued further. The results show that for both routes of administration there is no relationship between the vaginal response and body weight.

TABLE 2.

The response of mice, classified according to body weight, to a M.E.D. of oestrone for the subcutaneous and intravaginal routes of administration.

Subcutaneous			*Intravaginal*		
Body wt. (gm.)	Response Test 1	Test 2	Body wt. (gm.)	Response Test 1	Test 2
25	3:4*	4:4	18	1:1	1:1
26	6:6	5:6	19·5	2:3	0:3
27	3:3	5:6	20	2:8	5:8
28	9:13	10:13	20·5	1:5	3:5
29	7:9	7:9	21	4:8	1:8
30	10:19	8:19	21·5	4:7	3:7
31	7:12	6:12	22	5:18	8:18
32	3:11	4:11	22·5	11:20	13:20
33	7:13	5:13	23	8:18	5:18
34	3:12	2:12	23·5	16:37	15:37
35	7:13	8:13	24	12:29	11:29
36	2:6	4:6	24·5	16:29	21:29
37	6:11	6:11	25	20:36	17:36
38	11:15	12:15	25·5	27:49	30:49
39	4:8	4:8	26	19:45	24:45
40	2:3	0:3	26·5	21:42	21:42
41	6:9	3:9	27	20:45	19:45
42	1:4	3:4	27·5	15:31	18:31
43	3:8	6:8	28	17:30	18:30
44	0:2	0:2	28·5	13:28	12:28
45	0:1	0:1	29	6:17	11:17
47	2:4	3:4	29·5	12:18	10:18
49	1:2	1:2	30	6:13	5:13
50	1:1	0:1	30·5	11:16	10:16
53	1:1	1:1	31	2:5	4:5
55	0:1	0:1	31·5	2:5	3:5
57	0:1	0:1	32	0:1	0:1
			33	4:4	2:4
			33·5	0:2	0:2
			34	0:1	0:1
Total animals: 195			Total animals: 571		

* The response is expressed as the ratio of the number of animals positive to the number in the group.

TABLE 3.

Analysis of the data of Table 2. (b is the linear regression of probit response on body weight in gm. units, and s_b its standard error.)

	Subcutaneous		*Intravaginal*	
	b	s_b	*b*	s_b
Test 1	—0·0273	0·0152	0·0168	0·0194
Test 2	0·0256	0·0155	0·0216	0·0196

Analysis of the data of Marrian and Parkes (1929).

	Subcutaneous	
	b	s_b
Test 1 (Table IV)	—0·0376	0·0249
Test 2 (Table V)	—0·0548	0·0379

It is of interest to examine further the data given by Marrian and Parkes (1929) for the subcutaneous route using these methods of analysis, since they concluded that a small effect may be present. The regression coefficients are appended to Table 3, and it is seen that the negative regression coefficients are not significant, although if they are independent estimates the weighted mean regression is of borderline significance. The present evidence from subcutaneous tests gives no indication of any such trend, and since the work is based on about 3·5 times as much *information* (Fisher, 1948) as that of Marrian and Parkes, it seems that no relationship exists between body weight and vaginal response.

As an alternative approach to this problem, the weights of animals responding similarly in a number of tests may be compared. This method has been used by Emmens (1939) and further data are provided for analysis by the present work. The results are shown in Table 4 for both subcutaneous and intravaginal administration, where no differences are found.

TABLE 4.

The mean weights and their standard errors (S.E.) of mice which respond (1) positively, (2) negatively in three successive tests.

	Subcutaneous		*Intravaginal*	
Response	Mean wt.	S.E.	Mean wt.	S.E.
+ + +	32·9	5·0	26·4	2·7
— — —	35·6	6·9	25·6	3·0
	$t_{(74)} = 0·32$; $P > 0·05$		$t_{(212)} = 0·20$; $P > 0·05$	

Body weight and the mode of collection of colonies. The mean weight of the 30 sub-groups of mice in Colony III on the 7th October, 1952, is given in Table 5. At this stage the youngest mice (group 10) were 9 weeks old and the oldest mice (group 1) were 19 weeks old. The analysis of variance shows that the mice of classes A and B (both ovariectomized at 6 weeks) are similar, both being

significantly heavier than Class C. This is a well-known effect of ovariectomy, due to the deposition of adipose tissue. The fortnightly priming of class A had no apparent effect in combating the effect of ovariectomy on body weight.

TABLE 5.

The mean body weights of mice in each subgroup of Colony III.

Group No.	*Mean body weight (gm.)* A	B	C
1	24·6	25·7	23·8
2	24·6	25·8	23·3
3	26·4	26·1	24·8
4	26·0	24·6	24·0
5	25·0	26·1	25·2
6	25·9	26·1	22·9
7	26·2	26·5	24·5
8	23·8	24·8	24·2
9	26·6	24·2	23·5
10	24·3	23·3	22·7
Mean class body weight:	25·3	25·3	23·9

ANALYSIS OF VARIANCE.

Source of variation	D.f	Sum of squares	Mean square	F	P
Between classes	(2)	(139)			
A v B	1		0	<1	$>0{\cdot}05$
A + B v C	1		139	22·1	$<0{\cdot}001$
Between groups	(9)	(130)			
Linear regression	1		20	3·2	$>0{\cdot}05$
Deviations	8		14	2·2	$>0{\cdot}05$
Error	293	1843	6·3		

Effect of mode of collection of colonies on response.

A comparison of dose-response lines obtained with classes A, B and C. The three classes of Colony III were used in this work. Table 6 contains the design, results and analysis of a two-factor experiment (Test I in Table 1). This experiment is conveniently analysed by the angular transformation and the analysis of variance (see Claringbold, Biggers and Emmens, 1953). Comparison of the error mean square with the theoretical variance indicates no significant difference, implying that all three dose-response lines are parallel, and that the theoretical variance may be used for testing the significance of other mean squares. The analysis of variance indicates that no differences exist between the response of animals of classes A, B and C.

The response of classes A, B and C to two M.E.D.s. The response of each animal was determined on two occasions, 14 days apart (Tests II and III, in Table 1), and is summarized in Table 7. These data were analysed following

the angular transformation, which can be used provided there are no regions of 0 p.c. and 100 p.c. responses. The response to Tests II and III are not statistically independent and are analysed separately.

TABLE 6.

The effect of three levels of oestrone administered intravaginally on the three classes A, B and C (30 animals per group). The response is shown as the number of animals positive.

Class	Dose oestrone (10^{-4} μg.)		
	1·5	*3*	*6*
A	5	8	19
B	5	14	20
C	8	17	16

ANALYSIS OF VARIANCE (Angular transformation).

Source of variation	D.f	Sum of squares	Mean square	F	P
Between classes	(2)	(65)			
A v B	1		33	1·2	$>0·05$
A + B v C	1		32	1·2	$>0·05$
Between doses	(2)	(971)			
Linear	1		963	34·0	$<0·001$
Quadratic	1		8	<1	$>0·05$
Error	4	170	43	1·5	$>0·05$
Theoretical variance	∞		28		

TABLE 7.

The response of the subgroups of Colony III to two successive M.E.D.s (Tests II and III). The response is expressed as the proportion responding to the number per group.

Group	Tests II			Tests III		
	A	B	C	A	B	C
1	5:*10*	4:*10*	6:*12*	4:*10*	3:*10*	6:*12*
2	5:*11*	2:*8*	4:*11*	5:*11*	2:*8*	5:*11*
3	4:*11*	7:*10*	3:*11*	6:*11*	5:*10*	2:*11*
4	5:*7*	5:*8*	4:*8*	6:*7*	4:*8*	5:*8*
5	8:*10*	5:*8*	6:*10*	4:*10*	4:*8*	7:*10*
6	5:*9*	4:*7*	4:*13*	6:*9*	3:*7*	3:*13*
7	7:*9*	7:*10*	7:*12*	6:*9*	5:*10*	7:*12*
8	5:*12*	7:*12*	6:*12*	4:*12*	4:*12*	5:*12*
9	7:*11*	4:*12*	3:*11*	4:*11*	4:*12*	9:*11*
10	6:*10*	4:*9*	5:*11*	5:*10*	5:*9*	8:*11*
Weighted mean response:		50·67 p.c.			47·50 p.c.	
Significance of the weighted deviations from the means:		$F_{(29,\infty)} = 0·99$; $P > 0·05$			$F_{(29,\infty)} = 1·38$; $P > 0·05$	

For convenience a group size of 10 was given unit weight and the weighted mean response determined. This was then transformed back to a percentage value (see Table 7). The weighted sum of squares of deviations from the mean, divided by the number of degrees of freedom, gives the estimate of observed variance. With group size 10 the theoretical variance of a mean value is 820·7/10. Comparison of the observed and theoretical variances may be made by the F test of significance. The two values of F are shown in Table 7 and are not significant, indicating that any differences between groups and classes are those of random sampling.

DISCUSSION.

Body weight.

Several investigators have considered the effect of body weight on the vaginal response of ovariectomized mice or rats to the subcutaneous injection of oestrogen. Bugbee and Simond (1926) corrected their results to allow for variations in body weight, while Coward and Burn (1927) found in the rat that body weight was of no consequence. In more detailed examinations in mice (Marrian and Parkes, 1929; Emmens, 1939) no significant effects were found although it was suggested that a small degree of relationship exists. The work described in this paper has failed to demonstrate any relationship between body weight and the response to oestrogens for subcutaneous administration. Taken as a whole, therefore, the work of all investigators shows that with subcutaneous administration there is no relationship between vaginal response and body weight. Our own data for intravaginal administration have also shown no relationship. Thus "corrections" of dose for body weight will lead to additional variability and should not be used with these techniques.

Priming.

In the past many workers have gone to considerable trouble over the preparation of mice for the assay of oestrogens by the Allen-Doisy method, the object being to increase "accuracy." For example, Kahnt and Doisy (1928), in reply to criticism of the technique introduced by them in 1924, proposed the following method:

> Daily smears were made for 3 weeks before ovariectomy in order to select animals with normal cycles. Following ovariectomy daily smears were made for a further 2 weeks and animals showing oestrous or pro-oestrous smears later than 2 days after ovariectomy were rejected. The animals were then primed with 2 R.U.; one week later they were tested with 1·3 R.U.; and one week later still were again tested with 0·7 R.U. Those not responding to the first test and those responding to the second were rejected. An assay was never conducted on animals unless they had responded positively to a dose one week before.

Palmer (1937, 1938, 1941) has reiterated these ideas and uses only mice which have responded positively to a test or special dose one week before. This procedure is justified by observations suggesting that previous treatment condi-

tions subsequent responses, a finding not substantiated by Emmens (1939) or Biggers, Claringbold and Emmens (1954) in both subcutaneous and intravaginal tests. Further, the results of this paper show that three very different methods for the preparation of colonies produce no differences in response to oestrone administered intravaginally. The simple procedure of collecting the animals over a wide range of ages, ovariectomizing them together and giving a single priming treatment, seems all that is necessary for the establishment of a homogeneous colony. Emmens (1950b), however, has shown the necessity of priming every 6 to 8 weeks subsequently to maintain sensitivity.

Other aspects of the above work, such as the rejection of animals with low and high sensitivity, are discussed elsewhere (Biggers, Claringbold and Emmens, 1954).

SUMMARY.

The vaginal response to oestrone administered either intravaginally or subcutaneously is not related to body weight.

In order to produce a large homogeneous colony of ovariectomized mice, the simple procedure of collecting the required number, ovariectomizing them together and then giving a single priming treatment seems all that is required.

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THE TOXICITY OF SOME ANTIBACTERIALS FOR BULL, RAM, RABBIT AND HUMAN SPERMATOZOA

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Mammalian semen when collected by the artificial vagina or by electrical stimulation (Gunn, 1936) usually contains bacteria which may come from the urogenital tract or the external environment (Gunsalis, Salisbury and Willett, 1941; Edmondson, Tollman and Herman, 1949; Almquist, Prince and Reid, 1949). Seminal plasma and semen diluents are excellent media for bacteria, which proliferate rapidly, particularly if the semen is not stored at low temperatures (Hammond, 1930; Foote and Bratton, 1950). Bacterial growth can, for example, be sufficiently great at 37° C. to cause an increase in the oxygen consumption of ram and rabbit semen within five hours (White, 1953).

From the practical viewpoint, the growth of bacteria in semen used for artificial insemination is undesirable because of the risk of transmitting genital infection and the harmful effect which some bacteria may have on spermatozoa (Edmondson, Tollman and Herman, 1941). The former problem was recognized very early by Ivanov (1917), who investigated the control of infection by adding such substances as ethyl alcohol, atoxyl and salvarsan to contaminated semen.

Following the observation of Shettles (1940) that the sulphonamides were not toxic to human spermatozoa in bacteriostatic concentrations, Knodt and Salisbury (1946) introduced them into artificial insemination practice for the control of bacterial growth in bull semen. Since then numerous studies have been made on the effect of the sulphonamides and antibiotics on bacteria in bull semen and on the motility and fertility of bull spermatozoa (for review see Easterbrooks, 1951). Little information, however, is available on the effect of antibacterials on the spermatozoa of other species and it is the purpose of this paper to present a comparative study of the toxicity of sulphanilamide, penicillin, streptomycin, aureomycin and chloromycetin to ram, bull, rabbit and human spermatozoa.

Some experiments on the metabolic effects of toxic concentrations of aureomycin and chloromycetin are also included.

Materials and Methods.

Semen.

Ram semen was obtained by electrical ejaculation (Gunn, 1936); bull and rabbit semen was collected in an artificial vagina, and human semen was obtained from a Sterility Clinic. In all cases, only normal ejaculates of good motility were employed, and were used singly.

Experiments with ram and rabbit semen were started immediately after collection and those with bull semen within 2 hours of collection; in the latter case, the semen was slowly cooled to about 10° C. and kept at this temperature during transport to the laboratory. The human semen was between 4 and 6 hours old before use.

Techniques.

Semen was diluted 1 in 40 in small tubes for motility observations, and 1 in 10 in Warburg flasks for metabolic studies. Both types of experiment were done at 37° C.

Motility was scored at hourly intervals over a 4-hour period by the system of Emmens (1947). Full motility was rated at 4 and complete immotility as zero, but in presenting results the actual scores have been multiplied by 4, since quarter-grades were frequently used.

Oxygen uptake was measured at hourly intervals for two hours by the direct Warburg technique (Umbreit, Burris and Stauffer, 1949). The shaking rate was 114 strokes/min. and the gas phase air.

Lactic acid was estimated by the method of Barker and Summerson (1941) at the start and end of experiments and the total lactic acid production over the 2-hour period calculated.

After the metabolic experiments, spermatozoal counts were made in duplicate from the flasks, using a haemocytometer.

Diluents and antibacterials.

An isotonic diluent of pH = 7·0 was used in all experiments and had the following composition:

0·032 M—$NaH_2PO_4H_2O$, 0·048 M—$Na_2HPO_412H_2O$, 0·036 M—NaCl, 0·004 M—KCl, 0·022 M—fructose.

The antibacterials employed were:

Sulphanilamide (pure): Elliotts & Australian Drug Pty. Ltd.

Chloromycetin (pure): Parke, Davis & Co. Ltd.

Penicillin (cryst.): Glaxo Laboratories Ltd.; Commonwealth Serum Laboratories (1 μg. = 1 unit).

Dihydrostreptomycin sulphate: Glaxo Laboratories Ltd.; Rhône Poulenc.

Aureomycin hydrochloride: Lederle Laboratories. The aureomycin was filtered to remove excipient (titanium dioxide).

Statistical treatment.

Where responses to treatment have been virtually of the all-or-none type (as with 5,000 μg. chloromycetin/ml.) statistical analysis has been considered unnecessary. In other cases the *t*-test has been applied using the standard error of the mean of differences between the treated sample and the control from the same ejaculate.

The *P* values given in the text are for 3 degrees of freedom in all cases.

RESULTS.

Motility observations.

Preliminary tests with ram and bull spermatozoa indicated that penicillin, streptomycin, chloromycetin and sulphanilamide in concentrations of 200 μg./ml. were not toxic. Aureomycin, on the other hand, seemed more spermicidal, although 20 μg./ml. was quite well tolerated.

More systematic experiments were then undertaken in which the concentrations were increased fivefold and twenty-five fold, giving three levels for each antibacterial. Each treatment was replicated on four bull, ram, rabbit and human ejaculates, respectively, with a common control for each ejaculate. The total motility scores for each species are shown in Tables 1, 2, 3 and 4.

TABLE 1.

The total motility score (× 4) of bull spermatozoa over a 4-hour period at 37° C. in phosphate-fructose diluents containing antibacterials.

Antibacterial	Concentration μg./ml.	Ejaculate 1	2	3	4	Mean
Nil	—	64	58	48	50	55
Penicillin	5,000	63	63	61	60	62
	1,000	65	64	58	53	60
	200	63	63	56	50	58
Streptomycin	5,000	63	55	45	52	54
	1,000	63	60	52	48	56
	200	62	60	55	49	57
Aureomycin	500	15	4	17	6	11
	100	61	46	56	62	56
	20	60	60	55	52	57
Chloromycetin	5,000	1	1	0	0	1
	1,000	48	58	50	47	51
	200	65	52	50	40	52
Sulphanilamide	5,000	55	53	51	46	51
	1,000	62	55	50	43	53
	200	64	60	53	45	51

TABLE 2.

The total motility score (× 4) of ram spermatozoa over a 4-hour period at 37° C. in phosphate-fructose diluents containing antibacterials.

Antibacterial	Concentration μg./ml.	Ejaculate 1	2	3	4	Mean
Nil	—	50	66	80	68	66
Penicillin	5,000	46	63	80	76	66
	1,000	50	66	76	68	65
	200	45	55	80	62	61
Streptomycin	5,000	26	17	60	59	41
	1,000	50	66	78	78	68
	200	54	58	72	80	66
Aureomycin	500	28	19	11	15	18
	100	52	20	65	65	51
	20	50	64	80	80	69
Chloromycetin	5,000	8	8	0	2	5
	1,000	47	53	80	72	63
	200	51	61	78	66	64
Sulphanilamide	5,000	50	70	54	68	61
	1,000	50	70	76	68	66
	200	44	69	78	66	64

TABLE 3.

The total motility score (× 4) of rabbit spermatozoa over a 4-hour period at 37° C. in phosphate-fructose diluents containing antibacterials.

Antibacterial	Concentration μg./ml.	Ejaculate 1	2	3	4	Mean
Nil	—	34	49	35	48	42
Penicillin	5,000	47	33	38	49	44
	1,000	60	24	40	49	43
	200	60	51	41	50	51
Streptomycin	5,000	38	30	28	43	35
	1,000	45	44	32	45	42
	200	57	43	39	45	46
Aureomycin	500	39	20	22	38	30
	100	45	43	34	36	40
	20	58	51	36	46	48
Chloromycetin	5,000	0	0	0	0	0
	1,000	25	33	14	20	23
	200	50	46	35	47	45
Sulphanilamide	5,000	50	30	37	57	46
	1,000	48	52	41	40	45
	200	46	26	36	48	39

TABLE 4.

The total motility score (× 4) of human spermatozoa over a 4-hour period at 37° C. in phosphate-fructose diluents containing antibacterials.

Antibacterial	Concentration μg./ml.	Ejaculate 1	2	3	4	Mean
Nil	—	40	50	40	20	38
Penicillin	5,000	30	46	36	18	33
	1,000	40	50	40	20	38
	200	40	50	38	20	37
Streptomycin	5,000	42	44	34	20	35
	1,000	42	49	37	20	37
	200	40	50	38	20	37
Aureomycin	500	20	47	12	0	20
	100	31	50	31	12	31
	20	42	50	38	20	38
Chloromycetin	5,000	0	4	1	0	1
	1,000	8	44	9	0	15
	200	29	49	32	14	31
Sulphanilamide	5,000	8	34	10	6	15
	1,000	26	50	34	18	32
	200	34	50	40	23	37

Penicillin. Penicillin was not toxic to bull, ram, rabbit or human spermatozoa even at the highest concentration (5,000 μg./ml.).

Sulphanilamide. The motility of human spermatozoa was significantly depressed by the highest concentration of sulphanilamide (5,000 μg./ml.), usually within one hour ($t = 5{\cdot}0$; $P < 0{\cdot}05$); but the lower concentrations were harmless. At no concentration did sulphanilamide significantly affect bull, ram or rabbit spermatozoa.

Streptomycin. Streptomycin at the highest concentration (5,000 μg./ml.) depressed the motility of all four ram ejaculates in one to four hours. However, the variability in response was such that the t value does not quite reach significance at the 5 p.c. level. It was innocuous, in all concentrations tried, to bull, rabbit and human spermatozoa.

Aureomycin. Aureomycin was significantly toxic at the highest level (500 μg./ml.) to bull ($t = 9{\cdot}1$; $P < 0{\cdot}05$), rabbit ($t = 24{\cdot}0$; $P < 0{\cdot}01$) and human ($t = 3{\cdot}4$; $P < 0{\cdot}05$) spermatozoa. Bull spermatozoa were usually rendered immotile within an hour, but the motility of rabbit and human spermatozoa was not appreciably depressed for 2 to 4 hours.

Aureomycin was more toxic to ram spermatozoa and not only significantly depressed motility at 500 μg./ml., usually within an hour ($t = 4{\cdot}9$; $P < 0{\cdot}05$), but also tended to reduce motility at 100 μg./ml.

Chloromycetin. The highest concentration of chloromycetin (5,000 μg./ml.) was almost instantaneously spermicidal to all species. Lower levels did not significantly affect the motility of bull and ram spermatozoa but 1,000 μg./ml. was significantly toxic to rabbit ($t = 4{\cdot}6$; $P < 0{\cdot}05$) and human ($t = 3{\cdot}7$; $P < 0{\cdot}05$) spermatozoa in one to three hours.

Metabolic studies.

The effect was studied of the two most consistently toxic antibiotics (chloromycetin and aureomycin) on the metabolism of bull and ram spermatozoa.

Tables 5 and 6 show the progressive hourly oxygen uptake over a 2-hour period of bull and ram spermatozoa respectively in the presence of 500 μg. aureomycin/ml. and 5,000 μg. chloromycetin/ml. The spermatozoa of both species showed little, or no, respiratory activity when treated with chloromycetin. Aureomycin, however, had a differential effect, since it consistently depressed the oxygen consumption of bull spermatozoa ($t = 5{\cdot}4$; $P < 0{\cdot}05$), but increased that of ram spermatozoa ($t = 11{\cdot}2$; $P < 0{\cdot}01$).

TABLE 5.

The progressive hourly oxygen uptake of 10^8 bull spermatozoa in the presence of high concentrations of aureomycin and chloromycetin.

Ejaculate	Hours	Control (μl.)	Aureomycin (500 μg./ml.)	Chloromycetin (5,000 μg./ml.)
1	1	9·9	5·1	0·7
	2	18·1	7·1	1·1
2	1	6·7	5·8	0·3
	2	12·8	8·4	1·1
3	1	7·8	3·8	0·3
	2	15·0	5·9	1·6
4	1	9·6	4·1	0·5
	2	19·2	7·1	2·6

TABLE 6.

The progressive hourly oxygen uptake of 10^8 ram spermatozoa in the presence of high concentrations of aureomycin and chloromycetin.

Ejaculate	Hours	Control (μl.)	Aureomycin (500 μg./ml.)	Chloromycetin (5,000 μg./ml.)
1	1	17·4	30·6	0·0
	2	28·2	49·1	0·0
2	1	14·1	24·4	0·9
	2	22·8	42·3	1·5
3	1	28·4	38·4	1·2
	2	45·1	70·7	2·4
4	1	17·2	22·4	3·5
	2	24·7	41·4	4·7

The corresponding total lactic acid production of bull and ram spermatozoa is set out in Table 7. Chloromycetin significantly depressed the glycolysis of both bull and ram spermatozoa ($t = 6·3$; $P < 0·01$ and $t = 4·4$; $P < 0·05$ respectively). These spermatozoa again showed a differential response to aureomycin, the glycolysis of bull spermatozoa being stimulated ($t = 4·1$; $P < 0·05$) and that of ram spermatozoa, depressed ($t = 3·9$; $P < 0·05$).

TABLE 7.

Micrograms of lactic acid produced by 10^8 bull and ram spermatozoa in the presence of high concentrations of aureomycin and chloromycetin.

Species	Ejaculate	Control	Aureomycin (500 μg./ml.)	Chloromycetin (5,000 μg./ml.)
Bull	1	85	120	1
	2	85	136	50
	3	103	127	26
	4	58	134	—9
	Mean	83	129	17
Ram	1	167	12	—3
	2	96	64	12
	3	305	132	18
	4	152	27	—45
	Mean	180	59	—5

DISCUSSION.

The finding that 5,000 μg./ml. of sulphanilamide and penicillin is not toxic for bull spermatozoa is in agreement with the results of Knodt and Salisbury (1946), and of Sykes and Mixner (1951). Other workers, however, claim that motility is depressed by 1,000 units/ml. after 20 days at 5° C. (Almquist, Thorp and Knodt, 1948; Myers and Almquist, 1951). Foote and Salisbury (1948) found a great difference between the toxicity of two commercial samples of penicillin; one was toxic in a concentration of 63 units/ml. and the other innocuous at 800 units/ml. over a 16-day period at 5° C. Different batches of penicillin

from two manufacturers were used here, and all were uniformly harmless. Sykes and Mixner (1951) have also been unable to detect any difference in the toxicity of different brands and lots of penicillin.

Over much longer periods than used here (14 or 20 days) concentrations of streptomycin above 1,000 μg./ml. may depress motility (Almquist, Glantz and Thorp, 1948; Myers and Almquist, 1951) although Sykes and Mixner (1951) have been unable to confirm this.

Aureomycin and chloromycetin were much more toxic to bull spermatozoa than were the other antibacterials. Nevertheless, concentrations that are bacteriostatic (< 100 μg./ml. according to Bliss and Todd, 1949) were well tolerated in these experiments. Foote and Bratton (1950) also found 100 μg./ml. of aureomycin innocuous but Myers and Almquist (1951) report that over long periods (14 days) 50 μg./ml. depresses the motility of bull spermatozoa. Sykes and Mixner (1951) put the toxic level of aureomycin and chloromycetin in the range 125–250 μg./ml. and 1,000–2,000 μg./ml., respectively. In view of their wide antibacterial spectrum, both new antibiotics merit trial for controlling bacterial growth in semen for artificial insemination.

The mode of action of chloromycetin apparently differs in spermatozoa and bacteria, since chloromycetin has no effect on the respiration of bacteria (Smith, Worrel and Swanson, 1949) whereas in the experiments reported here, it greatly reduced both respiration and glycolysis.

More surprising is the differential effect of high concentrations of aureomycin on the metabolism of ram and bull spermatozoa. Aureomycin (500 μg./ml.) clearly stimulated the respiration of ram spermatozoa at the expense of glycolysis but has the reverse effect on the metabolism of bull spermatozoa; and yet it depresses motility in both instances at this concentration.

SUMMARY.

In studies of the motility of diluted bull, ram, rabbit and human spermatozoa over a 4-hour period at 37° C. it was found that:

Penicillin was not toxic at up to 5,000 μg./ml. to the spermatozoa of any species.

Streptomycin at 5,000 μg./ml. tended to depress the motility of ram spermatozoa but did not affect the motility of the others.

Sulphanilamide at 5,000 μg./ml. adversely affected human spermatozoa but 1,000 μg./ml. was innocuous. No other effects were seen.

Aureomycin was tolerated by the spermatozoa of all species in concentrations up to 100 μg./ml. Five times this concentration was toxic in each case.

Chloromycetin was innocuous to ram and bull spermatozoa in concentrations up to 1,000 μg./ml. and to rabbit and human spermatozoa at a level of 200 μg./ml. A fivefold increase in concentration was again toxic in each case.

Metabolic studies over 2 hours at 37° C. showed that:

Chloromycetin at 5,000 μg./ml. greatly depressed the respiration and glycolysis of ram and bull spermatozoa.

Aureomycin at 500 μg./ml. stimulated the respiration of ram spermatozoa and depressed glycolysis, but had a reverse effect in each case on bull spermatozoa.

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THE POINT OF THE INHIBITORY ACTION OF THE IODOACETYL RADICAL ON PHOTOSYNTHESIS

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Various investigators (Kohn, 1935; Nakamura, 1938; Gaffron, 1945; Macdowall, 1949; Calvin *et al.*, 1951; Ehrmantraut and Rabinowitch, 1952) have examined the inhibitory effect of the iodoacetyl radical (as iodoacetic acid or iodoacetamide) on photosynthesis or on partial reactions probably involved in it. The evidence provided by these studies as to its point of action on photosynthesis is, however, either inadequate or contradictory. For example, Macdowall (1949) suggested it affected one of the reactions involved in photosynthesis which take place within the chloroplast on the basis of a 50 p.c. depression by 0·01 M iodoacetic acid of the rate of reduction of phenol indophenol by illuminated spinach chloroplasts. However, it would appear that some other reaction in the photosynthetic process is much more sensitive to the iodoacetyl radical since Kohn (1935), Nakamura (1938), Gaffron (1945) and Calvin *et al.* (1951) reported that much lower concentrations of this, viz. *ca.* 10^{-4} M, produced at least as great a depression of the whole photosynthetic process in *Chlorella* and *Scenedesmus*.

Comparisons of effects on different organisms are, of course, open to question in view of the possibility of different concentrations of particular enzymes, etc. Moreover, the possibility that there may be more than one point of action of the iodoacetyl radical on photosynthesis cannot be disregarded, particularly in view of recent suggestions (Arnon, 1952; Calvin and Barltrop, 1952) of the participation in photosynthesis of at least two different reactions possibly sensitive to this reagent (Barron, 1951). Therefore, in this paper the results are presented of an investigation in which the effect of the iodoacetyl radical on the whole photosynthetic process of *Chlorella pyrenoidosa* is compared with its effect on the partial photosynthetic reaction involved in the evolution of oxygen accompanying the reduction of p-benzoquinone by illuminated cells of this organism (Rabinowitch, 1952; Fraser and Briggs, 1953).

Materials and Methods.

A strain of *Chlorella pyrenoidosa*, No. 211-8a in the Cambridge Botany School Algal Culture Collection, kindly supplied by Dr. Pringsheim, was used throughout this investigation.

Material for experiments was grown in pure culture in liquid medium of the following composition:

KNO_3–0·025 M	$FeSO_4.7H_2O$–0·00002 M
$MgSO_4.7H_2O$–0·020 M	Potassium citrate–0·00002 M
KH_2PO_4–0·018 M	

Trace elements: B, 0·05 p.p.m.; Mn, 0·5 p.p.m.; Zn, 0·07 p.p.m.; Cu, 0·002 p.p.m.; Mo, 0·02 p.p.m. (*cf.* Warburg and Negelein, 1922; Winokur, 1948). Glass distilled water was used throughout.

The continuously shaken cultures were grown at 25° C. with continuous illumination (4,000 lux) and a continuous supply of 4 p.c. carbon dioxide in air. The cells were used for experiments after six days' growth from a 15 c.mm. inoculum taken from a stock culture kept in the refrigerator. The cells were separated from the culture medium on the centrifuge and then washed twice in the buffer used in the experiments.

Measurements of oxygen evolution in photosynthesis and in the reduction of p-benzoquinone were made at 25° C. using standard Warburg manometric techniques (Warburg, 1919; Umbreit, Burris and Stauffer, 1949). The quotient CO_2/O_2 for photosynthesis under acid conditions (pH 4·6, 4 p.c. CO_2 in air as gas phase) was determined by the indirect (two vessel) method (Warburg, 1924; Umbreit, Burris and Stauffer, 1949) with due regard to the conditions for accurate measurement (Nishimura, Whittingham and Emerson, 1951). Using the average figure of —0·90 for this quotient, oxygen evolution in photosynthesis under acid conditions was measured with only one vessel per treatment (Warburg and Negelein, 1922; Kohn, 1935). The correction necessary for oxygen uptake due to respiration was computed from measurements made in the dark after the period of photosynthesis. Since the correction was comparatively small and since respiration was only depressed by much higher concentrations of the inhibitors than was photosynthesis (*cf.* Kohn, 1935) such measurements were deemed sufficiently accurate for the approximate results desired in this investigation. Illumination was provided from below by 500-watt projection lamps giving a light intensity of about 40,000 lux at the level of the vessels. This light intensity was high enough to saturate all the systems studied.

The volume of cells used was determined by centrifuging aliquots of the suspension for fifteen minutes at about 3,000 r.p.m. in tubes with a calibrated length of capillary tubing at the bottom (*cf.* Sargent, 1940).

Most of the chemicals employed were of A.R. quality, while a few were B.D.H. standard reagents. Glass distilled water was used for all solutions and for the final rinsing of vessels, etc. Only a technical grade of p-benzoquinone was available but it was readily purified by double sublimation. Solutions of quinone in water are unstable in the light and were, therefore, freshly prepared for each experiment and kept in darkness as far as possible before use. The side arms of the vessels in which the quinone solution was placed before addition to the cell suspensions were enclosed in silver foil. Iodoacetamide was prepared from chloracetamide and sodium iodide by the method of von Braun (1908). It was recrystallized from water until a constant M.P. of 95° C. was obtained.

RESULTS.

Inhibition of photosynthesis by iodoacetic acid and iodoacetamide.

Acid conditions. Measurements of the effect of the inhibitors on photosynthesis under acid conditions, i.e. in pH 4·6 phosphate buffer with a saturating concentration (4 p.c.) of carbon dioxide in air as gas phase, were made using

one vessel per treatment after it was shown that the CO_2/O_2 quotient was not affected by the inhibitors. For example, in the presence of 0·000027 M iodoacetic acid which produced an approximately 80 p.c. depression of photosynthesis, the quotient was —0·91 over a 90-minute period while in the absence of iodoacetic acid it was —0·92.

As first noted by Kohn (1935) the degree of depression of photosynthesis by iodoacetic acid and iodoacetamide increased progressively with time after their addition over a fairly long period but eventually reached a steady value. Typical data for iodoacetic acid at pH 4·6 are shown in Fig. 1 and similar observations have been made with iodoacetamide under both acid and alkaline conditions. However, it was found that the same steady depressed rate of photosynthesis was attained much more rapidly if the inhibitor was added to the cells in the dark thirty minutes before illumination than if it was added while the illuminated cells were actively photosynthesizing (Fig. 1). Although the final degree of inhibition was the same, addition of the inhibitor in the dark beforehand shortened the time necessary to obtain figures for the steady state depressed rate of photosynthesis.

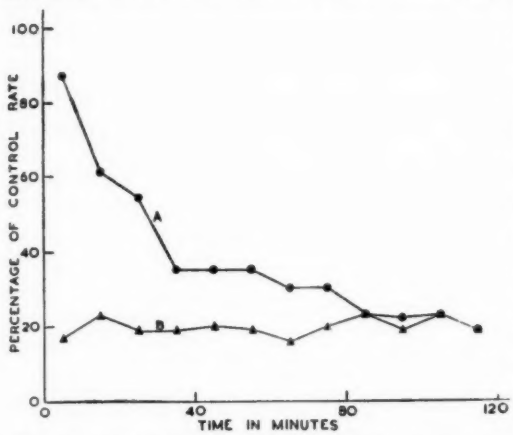

Fig. 1. Inhibition of photosynthesis by 0·000027 M iodoacetic acid at pH 4·6 with 4 p.c. carbon dioxide in air as gas phase.

Curve A, iodoacetic acid added at zero time.

Curve B, iodoacetic acid added half an hour before zero time in the dark.

The very low concentrations of iodoacetic acid and iodoacetamide which produced an approximately 50 p.c. depression of photosynthesis under acid conditions are given in Table 1. Much higher concentrations of these substances—0·00054 M iodoacetic acid and 0·0027 M iodoacetamide—were tested for comparison with their effects on the reduction of p-benzoquinone. They almost completely suppressed all measurable photosynthesis.

Alkaline conditions. Measurements of the effect of the inhibitors on photosynthesis under alkaline conditions were made with the cells suspended in the No. 9 carbonate-bicarbonate buffer (pH 9·4) devised by Warburg (1919) with the exception that the potassium rather than the sodium salts were employed. As Warburg (1919) first showed, this buffer provides a sufficiently high carbon dioxide concentration to saturate the photosynthetic system of Chlorella at the

saturating light intensity provided in these experiments. The very low concentration of iodoacetamide required to produce a 50 p.c. depression of photosynthesis in this buffer is shown in Table 1. Much higher concentrations such as 0·0027 M completely suppressed all measurable photosynthesis. The highest concentration of iodoacetic acid tested (0·01 M) was insufficient to produce more than a slight depression (10 p.c.) of the rate of photosynthesis.

TABLE 1.

The effect of iodoacetic acid and iodoacetamide on photosynthesis and the reduction of p-benzoquinone by Chlorella cells.

Process and conditions	Concentrations of inhibitor required to produce *ca.* 50 p.c. depression of rate	
	Iodoacetic acid	Iodoacetamide
Photosynthesis pH 4·6	0·000014 M	0·000027 M
Photosynthesis pH 9·4	>0·0108 M	0·000014 M
Quinone reduction pH 4·6	0·00054 M	>0·0027 M
Quinone reduction pH 6·5	0·0108 M	>0·0027 M

Inhibition of p-benzoquinone reduction by iodoacetic acid and iodoacetamide.

At pH 4·6. Since iodoacetic acid appeared to be a very effective inhibitor of photosynthesis at pH 4·6, its action, as well as that of iodoacetamide, on the reduction of p-benzoquinone was investigated at this pH. The cells were pretreated with the inhibitors in the dark for half an hour beforehand. Immediately upon illumination, 0·5 ml. of a solution containing 1 mg. of p-benzoquinone was added to the cells which were suspended in 4·5 ml. of pH 4·6 phosphate buffer. This concentration gives the maximum rate of oxygen evolution from quinone (Clendenning and Ehrmantraut, 1950; Fraser and Briggs, 1953) and the yields of oxygen were 75–80 p.c. of the theoretical. Carbon dioxide was excluded from the system by the presence of 0·2 ml. of a 10 p.c. solution of potassium hydroxide in the centre well of the vessel.

The concentration of iodoacetic acid required to produce an approximately 50 p.c. depression of the rate of oxygen evolution from p-benzoquinone at pH 4·6 over the first ten minutes is shown in Table 1. The rate was greatest during this period. The highest concentration of iodoacetamide which it was convenient to test, 0·0027 M, did not affect the rate of the oxygen evolution from p-benzoquinone.

At pH 6·5. For comparison with the results of Macdowall (1949) and Ehrmantraut and Rabinowitch (1952) obtained at pH 6·5, the effect of both inhibitors on the reduction of p-benzoquinone in pH 6·5 phosphate buffer was also examined. The concentration of iodoacetic acid required to produce an approximately 50 p.c. depression of the rate over the first ten minutes is shown in Table 1. Again, the highest concentration of iodoacetamide tested, 0·0027 M, did not affect the rate of oxygen evolution.

DISCUSSION.

The concentrations of the inhibitors found to produce a 50 p.c. depression of the photosynthesis of Chlorella are closely similar to those reported by Kohn (1935) for the same organism. As he also observed, the effect of iodoacetic acid was markedly dependent on the pH, a common complicating feature of the biological action of various weak acids and bases on intact cells (Simon and Beevers, 1952 a, b). The concentration of iodoacetic acid producing 50 p.c. depression of the rate of reduction of p-benzoquinone at pH 6·5 is also closely similar to that reported by Ehrmantraut and Rabinowitch (1952) for the same reaction and by Macdowall (1949) for phenol indophenol reduction by spinach chloroplasts. Here again the concentration required is markedly dependent on the pH. Since the effect of iodoacetamide is practically unaltered by pH, the results obtained with it are probably more suitable for comparisons of the effects of the iodoacetyl radical on different processes.

It is clear that under both acid and alkaline conditions photosynthesis is markedly inhibited by iodoacetamide but that the photochemical reduction of p-benzoquinone is not, at least by concentrations which almost completely inhibit photosynthesis. The situation is not quite so clear with iodoacetic acid but nevertheless at pH 4·6 the concentration required to produce a 50 p.c. inhibition of photosynthesis is only about one-fortieth of that required to produce a 50 p.c. inhibition of quinone reduction. Further, this latter concentration almost completely suppresses photosynthesis. These results render it unlikely that the inhibitory effect of low concentrations of the iodoacetyl radical on photosynthesis is due to an effect on the part of the photosynthetic mechanism concerned in the generation of reducing power and the liberation of oxygen, i.e. the splitting of water (Rabinowitch, 1952). It suggests rather that it affects some step in the transformations of carbon dioxide to carbohydrate or possibly a late stage in the transfer of hydrogen to carbon dioxide bound to some acceptor molecule (Rabinowitch, 1952). The results could be interpreted as indicating that iodoacetamide acts on this system more specifically than does iodoacetic acid.

As originally suggested by Kohn (1935) the inhibitory effect of the iodoacetyl radical on photosynthesis is probably due to combination with thiol groups necessary for the activity of some enzyme(s) concerned in the process (Barron, 1951). The relatively slow rate of reaction of the inhibitors with such groups (Barron, 1951) probably explains the fairly long exposure of the cells to the inhibitor necessary to attain steady depressed rate of photosynthesis. However, the much greater time required to produce the same depression when the inhibitors were added to cells actively photosynthesizing than when they were added to darkened cells some time before illumination suggests the possibility that the thiol groups are partially protected during photosynthesis in such a way as to reduce their rate of reaction with the inhibitor. Several instances have been

reported (Barron, 1951; Barron and Levine, 1952) of the protection of thiol groups essential for enzyme activity from reagents inactivating them by the presence of the substrate or coenzyme of the enzyme.

Such a variety of enzymes which could be involved in photosynthesis are known to require free thiol groups for their activity (Barron, 1951) that it is difficult to make any specific suggestion as to which, if any, of these might be involved in the depression of photosynthesis by low concentrations of the iodoacetyl radical. Although the observations of Calvin *et al.* (1951) initially appeared to exclude the possibility that a phosphoglyceraldehyde dehydrogenase sensitive to low concentrations of iodoacetamide was the enzyme concerned, more recent evidence and discussion presented by Arnon (1952) renders this a distinct possibility.

The inhibition of the reduction of p-benzoquinone by high concentrations of iodoacetic acid but not by iodoacetamide may be due to relatively unspecific combination of the former with enzyme proteins (Barron, 1951). However, the possibility of combination with thiol groups important in this reaction and present in relatively high concentrations or possessing a low affinity for the inhibitor cannot be excluded. Indeed, it is of particular interest in view of the recent suggestion by Calvin and Barltrop (1952) that a bi-radical formed by dissociation of a disulfide bond may be the form in which light energy absorbed and stored as electronic excitation in chlorophyll appears first as chemical bond potential energy.

It seems quite possible that the inhibition of photosynthesis by p-benzoquinone, ferricyanide or a mixture containing it, and benzaldehyde reported elsewhere (Clendenning and Ehrmantraut, 1950; Ehrmantraut and Rabinowitch, 1952; Fraser and Briggs, 1953) may be due to an effect on the same system attacked by the iodoacetyl radical since all these substances are capable of combining with or oxidizing thiol groups (Barron, 1951). This is supported by experiments which will not be described here in detail in which it was found that concentrations of benzaldehyde which caused a 50 p.c. depression of photosynthesis (Fraser and Briggs, 1953) did not decrease the rate of p-benzoquinone reduction.

SUMMARY.

The effect of iodoacetamide and iodoacetic acid on the photosynthesis of *Chlorella pyrenoidosa* has been compared with their effect on the photochemical reduction of p-benzoquinone by the same organism under the same experimental conditions. The former process was inhibited by concentrations of these substances which were much lower than those inhibiting the latter. These observations are interpreted as indicating that these substances do not inhibit photosynthesis primarily by inactivating the portion of this process concerned with the splitting of water and the evolution of oxygen.

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OBSERVATIONS ON COMPLEMENT FIXATION AND COMPLEMENT FIXATION INHIBITION USING CERTAIN AVIAN SERA

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The problems of the serological investigation of bird sera have received some attention since it was first realized that the complement fixation test was often not satisfactory with them.

Rice in 1948 showed that in systems using *Salmonella pullorum* and antisera from fowls, turkeys, ducks or geese, complement fixation did not occur. However, when antisera from these species were mixed with antigen, and complement and a known positive complement fixing serum were added to the system, no fixation took place. The bird sera had inhibited the fixation of complement by the known positive serum. Karrer, Meyer and Eddie (1950) showed, using systems with psittacosis virus and antisera, that not only did inhibition occur with fowl sera, but that also a percentage of sera from pigeons, birds which had previously been regarded as satisfactory for the complement fixation test, gave not complement fixation, but inhibition.

In investigations on the infection of birds with the virus of Murray Valley encephalitis, problems in the use of *in vitro* tests in systems where agglutination tests are impracticable have arisen, and have led to some further investigations on the complement fixation and inhibition reactions with bird sera.

MATERIALS AND METHODS.

The virus used in this investigation has been the South Australian strain of Murray Valley encephalitis virus. The antigens employed were lyophilized mouse brain antigens prepared by Mr. D. W. Howes according to technique previously described (Miles and Howes, 1952). The complement was lyophilized in this laboratory from large pools of guinea-pig serum. The haemolysin used was a commercial product bought from the Commonwealth Serum Laboratories. The techniques of the complement fixation and neutralization tests were those previously described by Miles and Howes (1952).

Complement fixation inhibition.

The results of using a system in which complement fixing and inhibiting sera were added to the system at the same time were found to be somewhat unreliable, and experiments were made to find the conditions under which the most satisfactory and reproducible results could

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be obtained when the unknown serum was allowed a preliminary period in which to react with antigen before the addition of complement and a known complement fixing serum. The differences between one hour's preliminary incubation at 37° C. and overnight at 4° C. were slight, but rather high titres were obtained by the latter technique in this system, and the technique described below is that which was finally adopted as being convenient and giving reasonably clear-cut and reproducible results.

Serial dilutions of the inactivated test serum were made in calcium magnesium saline beginning at 1: 5, and 2½ fixing doses of antigen were added. This mixture was incubated overnight at 4° C. and then left for one hour on the bench at room temperature. Complement was titrated to 50 p.c. haemolysis and 2½ 50 p.c. haemolytic doses were added to the system followed by twice the minimum dose of known positive serum required to give complete fixation under these conditions.

The whole was then incubated for one hour at 37° C. in a water bath. 3 p.c. sheep's red blood corpuscles sensitized with 5 minimal haemolytic doses of haemolysin were then added. The system was then returned to the water bath for 30 minutes. The tubes were centrifuged for 2 minutes at 2,000 r.p.m. to deposit remaining cells and the degree of haemolysis in the supernatant was read by comparison with appropriately diluted completely haemolysed tubes. The results were recorded as follows: 0 = complete haemolysis, 1 = more than 50 p.c. haemolysis, 2 = approximately 50 p.c. haemolysis, 3 = less than 50 p.c. haemolysis, 4 = no haemolysis. Only tubes recorded as 0 or 1 were regarded as positive. In this system care is needed in the selection of the known positive serum since it is essential that this serum should give no reaction with normal mouse brain. Provided a satisfactory positive serum is used the system can only test for reaction between the unknown serum and the virus part of the antigen.

It was hoped that under these conditions complement fixing sera might show low titre inhibition as some evidence has been obtained in this laboratory that this may occur with larger particle antigens, but in this system, with a small particle antigen, complement was fixed even when a positive serum had been in contact with antigen for 18 hours before the addition of the complement.

Birds.

The most detailed studies reported here have been made on homing pigeons. These birds are highly satisfactory for such studies since they survive well in the laboratory, and show no signs of ill-health when 7–10 ml. of blood are removed from a wing vein once or twice in the week for considerable periods. The first observations which preceded the more detailed studies were made on Indian doves *(Streptopelia suratensis)*; but the amount of blood which can be obtained from these birds without harming them allows only for a single test with no possibility of repetition. Some report will also be given of experiments on cockerels, a domestic duck and a white-backed magpie *(Gymnorhina hypoleuca)*.

EXPERIMENTAL.

During experiments on the infection of Indian doves with Murray Valley encephalitis and their subsequent development of antibodies (described in the following paper) certain anomalous serological results were obtained in the complement fixation and complement fixation inhibition tests. In view of the finding of Karrer, Meyer and Eddie in pigeons, it was thought likely that the reactions of this related species would also show some irregularity in behaviour. It was, however, surprising to find not only that there were differences between

different birds, but also that serum from the same bird taken on different occasions might behave differently.

Because it was not practicable, without killing the birds, to obtain sufficient blood from Indian doves to repeat any experiment or to do both neutralization and *in vitro* tests on the same specimen, it was decided to make a series of experiments on homing pigeons to try to explain this phenomenon.

Experiment 1.

Six adult pigeons were inoculated intradermally with approximately 50 mouse LD_{50} of virus. Blood taken at the time of inoculation did not neutralize significant amounts of the virus. Four of the birds were bled from a wing vein six hours after inoculation, and then daily for six days, and each blood was tested for the presence of virus. All showed a transient viraemia lasting between 24–48 hours. On the third and sixth day all birds had 7–10 ml. of blood removed and bleedings were then continued according to the schedule shown in Table 1 until 12 weeks after inoculation.

One of the pigeons not tested for viraemia failed to give serological evidence of infection and no results from this bird have been reported. No birds showed complement fixation or inhibition in blood taken on the third day, but three of the five showed inhibition at a titre of 1:5 on the sixth day, and all at titres from 1:10 to 1:80 on the 10th day. Pigeon 10 then gave a transient complement fixation on the 14th and 18th days; on the 14th day inhibition was absent in this bird, but could again be detected on the 18th day. No reaction could be detected after the 56th day (see Table 1). None of the other birds showed fixation at any stage and inhibition was last detected on either the 42nd or the 56th day.

Significant neutralization was not present in any serum on the 14th day, but the log. neutralization indices on the 42nd day varied between 1·2 and 2·5, and on the 84th day between 1·2 and 2·3.

Experiment 2.

Six pigeons which had been investigated previously for susceptibility to the virus and which had been inoculated either intracerebrally or intramuscularly with 10^8 mouse LD_{50} of virus, had been bled out on the 35th day of inoculation. Three of these birds had shown complement fixation and three inhibition. It was, on this account, thought likely that a larger dose of virus giving a more powerful antigenic stimulus might give a result different from that obtained in experiment 1.

Therefore, four of the pigeons used in experiment 1, and two further birds not previously used were inoculated intramuscularly with 10^9 mouse LD_{50} of virus. All birds looked ill for about 48 hours after inoculation, but from the third day onwards appeared normal. The results of serological testing are

TABLE 1.

Titres of serological tests in pigeon sera.

Days after inoculation 50 Mouse LD_{50} I.D.	Pigeon 7			Pigeon 8			Pigeon 9			Pigeon 10			Pigeon 11			Pigeon 13			Pigeon 14		
	*CFT	†CFI	‡NI	CFT	CFI	N1	CFT	CFI	N1	CFT	CFI	N1	CFT	CFI	N1	CFT	CFI	N1	CFT	CFI	N1
0			0			0			0			0			0						
3	0	0		0	0		0	0		0	0		0	0							
6	0	0		0	0		0	1:5		0	1:5		0	1:5							
10	0	1:40		0	1:40		0	1:40		0	1:80		0	1:20							
14	0	1:40	0·2	0	1:40	0·4	0	1:10	0·4	1:10	0	0·4	0	1:10	0·3						
18	0	1:40		0	1:80		0	1:20		1:10	1:10		0	1:20							
24	0	1:20		0	1:80		0	1:20		0	1:10		0	1:20							
30	0	1:20		0	1:40		0	1:10		0	1:10		0	1:10							
36	0	1:20		0	1:40		0	1:20		0	1:10		0	1:10							
42	0	1:10	2·5	0	1:20	2·2	0	1:5	1·2	0	1:5	1·5	0	1:10	2·4						
56	0	1:10		0	1:10		0	0		0	1:10		0	0							
70	0	0		0	0		0	0		0	0		0	0							
84	0	0	2·3	0	0	2·0	0	0	1·4	0	0	1·2	0	0							
91	Reinocultaed 10^9 Mouse LD_{50} I.M.															Inoculated 10^9 Mouse LD_{50} I.M.					
3	0	1:40		0	1:80		0	1:40		0	1:320					0	0	0	0	0	0
6	0	1:160		1:20	0		0	1:320		1:320**	1:320					0	0		0	1:20	
10	0	1:320		1:80§	1:160		0	1:320		1:320	0					0	0	0	1:20	0	
14	0	1:320	3·1	1:40	0		0	1:320	3·4	1:320	0	3·7				0	1:5	1·5	1:20	0	1·0
20	0	1:320		1:80	0		0	1:320		1:320	0					0	1:20		1:20	0	
28	0	1:160	3·3	1:20	0	3·0	0	1:320	2·3	1:160	0	3·5				0	1:20	2·0	1:20	0	1·5
35	0	1:160		1:40	0		0	1:320		1:320	0					0	1:40		1:10	0	
42							0	1:320		1:160	0					1:20**	1:40		1:10	0	
56	0	1:160		1:40§	1:160		0	1:80		1:160§	1:40					1:10**	1:40		0	1:10	
76	0	1:40	3·0	?**	1:80	3·7	0	1:80	3·9	1:160§	1:80	3·5				1:40**	1:40	2·8	0	0	1·8

* Complement fixation test. † Complement fixation inhibition test. ‡ Neutralization index. § Prozone present.

** Fixation was not complete in any tube.

recorded in the lower half of Table 1. All the re-inoculated birds were showing inhibiting antibody on the third day and two of them fixing antibody on the sixth day which, in these birds, replaced the inhibiting for several weeks.

In the two birds receiving the large primary inoculation no antibody was detected on the third day, but in one inhibiting antibody was present on the 6th day which changed to fixing antibody by the 10th day. This had reverted to inhibiting by the 56th day, but had disappeared by the 76th day. The other first showed inhibiting antibody on the 14th day and from the 42nd day to the 76th showed partial fixation as well as inhibition. Their log. neutralization indices were 1·0 and 1·5 on the 14th day, rising to 1·8 and 2·8 by the 76th day. The neutralization indices of all the re-inoculated birds were three logs. or higher at the end of the experiment.

The phenomenon reported by Karrer, Meyer and Eddie of sera which showed a prozone in the complement fixation test also giving a positive inhibition test was often observed in these birds, and it was noticed that this phenomenon was usual at the time when the type of reaction was changing.

TABLE 2.

Mixtures of serum from pigeons 9 and 10 taken 14 days after the second inoculation tested for complement fixation and inhibiting activity.

Type of serum and proportion			Serum dilutions								Normal antigen	Serum control	
CFT*	CFI†	Test	1:5	1:10	1:20	1:40	1:80	1:160	1:320	1:640	control	1:5	Titre
10	0	CFT	4	4	4	4	4	4	2	tr	0	0	1:320
		CFI	4	4	4	4	4	4	4	4		0	0
9	1	CFT	4	4	4	4	4	4	1	tr	0	0	1:160
		CFI	4	4	4	4	4	4	4	4		0	0
8	2	CFT	3	4	4	4	4	4	1	tr	0	0	1:160
		CFI	3	3	3	3	4	4	4	4		0	0
7	3	CFT	2	3	3	4	4	3	1	0	0	0	1:160
		CFI	0	0	0	0	2	3	4	4		0	1:40
6	4	CFT	1	1	2	3	3	2	tr	0	0	0	1:160
		CFI	0	0	0	0	0	2	3	4		0	1:80
5	5	CFT	tr	tr	1	2	3	2	tr	0	0	0	1:160
		CFI	0	0	0	0	0	0	3	4		0	1:160
4	6	CFT	tr	1	1	1	2	2	tr	0	0	0	?
		CFI	0	0	0	0	0	1	3	4		0	1:160
3	7	CFT	0	0	tr	1	1	1	0	0	0	0	0
		CFI	0	0	0	0	0	0	1	4		0	1:320
2	8	CFT	0	0	0	0	0	0	0	0	0	0	0
		CFI	0	0	0	0	0	0	1	3		0	1:320
1	9	CFT	0	0	0	0	0	0	0	0	0	0	0
		CFI	0	0	0	0	0	0	1	3		0	1:320
0	10	CFT	0	0	0	0	0	0	0	0	0	0	0
		CFI	0	0	0	0	0	0	0	3		0	1:320

* Complement fixation test. † Complement fixation inhibition.

Experiments on mixtures of pigeon sera.

In an attempt to gain more information about the phenomena observed in the investigations described, a series of experiments was made using mixtures of pairs of complement fixing and inhibiting sera either from the same or different birds.

TABLE 3.

Mixtures of serum from pigeon 8 taken 3 and 20 days after the second inoculation tested for complement fixing and inhibiting activity.

Type of serum and proportion			Serum dilutions							Normal antigen control	Serum control 1:5	Titre
CFT*	CFI†	Test	1:5	1:10	1:20	1:40	1:80	1:160	1:320			
10	0	CFT	4	4	4	4	4	3	1	0	0	1:160
		CFI	3	3	3	4	4	4	4		0	0
9	1	CFT	4	4	4	4	4	3	1	0	0	1:160
		CFI	3	3	3	4	4	4	4		0	0
8	2	CFT	4	4	4	4	4	2	1	0	0	1:160
		CFI	3	3	3	4	4	4	4		0	0
7	3	CFT	4	4	4	4	4	2	0	0	0	1:160
		CFI	3	3	3	4	4	4	4		0	0
6	4	CFT	4	4	4	4	3	2	0	0	0	1:160
		CFI	2	3	4	4	4	4	4		0	0
5	5	CFT	4	4	4	4	2	1	0	0	0	1:80
		CFI	2	3	3	3	4	4	4		0	0
4	6	CFT	4	4	4	3	3	1	0	0	0	1:80
		CFI	2	2	3	4	4	4	4		0	0
3	7	CFT	4	4	4	3	1	1	0	0	0	0
		CFI	2	2	3	3	4	4	4		0	0
2	8	CFT	3	4	3	1	1	0	0	0	0	1:20
		CFI	1	0	1	3	4	4	4		0	1:20
1	9	CFT	3	2	1	1	0	0	0	0	0	1:10
		CFI	0	0	0	1	4	4	4		0	1:40
0	10	CFT	1	0	0	0	0	0	0	0	0	0
		CFI	0	0	0	1	3	4	4		0	1:40

* Complement fixation test. † Complement fixation inhibition.

The two sera under test were mixed in varying proportions as shown in Tables 2 and 3. Serial dilutions of the mixtures were made and these were tested for complement fixing and inhibiting antibodies. In the small number of experiments made no two pairs of sera behaved exactly alike, but the results fell into two groups and typical results are shown in detail in Tables 2 and 3.

Table 2 shows the results of mixing sera from pigeons 9 and 10, both taken 14 days after the second inoculation, the former giving an inhibition titre of 1 : 320 and the latter a similar fixing titre. In this case inhibition was observed in all tubes containing a mixture of 3 or more parts of inhibiting serum in the total of 10 parts. Some evidence of fixation in the middle dilutions with a

very marked prozone was found from the series with 3 parts of fixing serum, but complete fixation in any tube was not seen until the mixture in which fixing serum was in excess by 7 parts to 3, and complete absence of prozone was found only where 9 parts of fixing serum were present.

Table 3 shows the results of mixing sera from pigeon 8 taken on the third and 20th days after the second inoculation. In this case the titres of the sera were not so high and in this test not so well balanced, but the change-over from fixation to inhibition was much more rapid and the amount of prozone seen was far less than with the other pair. The other pairs of sera either from different times in the same bird or on the same day in different birds fell between these extremes of pattern, except that in one mixture low titre inhibition was observed in three mixtures giving a substantial titre of complement fixation without any prozone.

Experiments on birds other than pigeons.

The experiments reported on birds other than pigeons were not primarily designed to investigate *in vitro* serological reactions, but rather infection with the virus and the serological investigations were not made so fully. Results are shown in Table 4. The two-week results are for investigations on the 14th or 15th day; the rest are given to the nearest whole week to enable the results to be shown on a single Table.

The evidence strongly suggests that Indian doves behave in the same way as pigeons in these tests, and, in fact, the results in the doves originally suggested that the pigeon experiments should be undertaken.

The cockerels, duck and white-backed magpie all showed inhibiting antibody; in the duck and magpie this lasted at least one year. One of the cockerels gave a transient low titre positive complement fixation test which is probably an unusual type of reaction for this species.

DISCUSSION.

The results reported in this paper suggest that there is a definite pattern of response to antigenic stimuli in pigeons. In experiment 1 the stimulus used was slight and may have approached the minimal. The reaction of four of the five birds which gave any response to this small stimulus was to produce relatively short-lasting complement fixation inhibiting antibodies of either moderate or low titre without ever giving complement fixation. The fifth bird gave a very transient positive complement fixation test having first given inhibition and reverting to inhibition afterwards. In all these birds relatively enduring neutralizing antibodies were also found.

The isolated observations on pigeons killed 35 days after infection with a large dose of virus suggested that a more intense stimulus might lead to a different pattern of response and consequently four of the original five birds were re-inoculated and two new birds were inoculated with a very large dose of

TABLE 4.

Titre of complement fixation and inhibition at times after inoculation in various birds.

Bird		Complement fixation test							Complement fixation inhibition						
		2 weeks	4	8	12	16	20	1 year	2 weeks	4	8	12	16	20	1 year
Indian Dove	6	1:160*	—†	1:160	—	1:20	1:40	—	1:80	—	0	—	0	0	—
„ „	7	1:40*	—	1:80	—	1:10	1:20	—	1:40	—	0	—	0	0	—
„ „	8	0	—	0	0	0	—	—	1:40	—	1:80	1:5	0	—	—
„ „	9	0	—	1:40	0	0	—	—	—	—	0	1:10	0	—	—
Cockerel	1	—	0	0	0	—	—	—	—	1:80	0	1:10	—	—	—
„	2	—	1:20	1:5	0	—	—	—	—	1:80	1:40	1:20	—	—	—
„	3	0	0	0	0	—	0	—	1:320	1:160	1:40	1:40	—	1:40	—
„	5	0	0	0	0	—	0	—	1:160	1:80	1:80	1:80	—	0	—
Duck		—	0	0	0	—	0	0	—	1:80	1:40	1:20	—	1:20	1:20
Magpie		—	0	0	0	—	0	0	—	1:80	1:40	1:80	—	1:80	1:40

* Prozone present. † Not tested.

virus. The re-inoculated birds, which had given negative tests for at least three to five weeks before the second inoculation, gave a prompt secondary response, all showing substantial inhibiting titres in three days, and two of the four going over to fixing antibodies by the sixth day. The other two did not show fixation at any time. The two birds receiving the large primary inoculation both showed the time intervals of the primary response and neither gave very high titres, but both did give some complement fixation after first giving inhibition and one had reverted to inhibition and then lost all demonstrable antibody before the experiment was terminated 76 days after infection.

The results reported by Karrer, Meyer and Eddie on the antibodies to psittacosis virus in pigeons showed some birds giving fixation and some inhibition. The antigenic stimulus from psittacosis virus in these birds is certainly long-lasting compared to the brief stimulus given by Murray Valley encephalitis and probably corresponds better to the intense than to the minimal stimulus given in these experiments. The experience in this laboratory is that pigeons give positive tests for more than two years after infection with psittacosis viruses.

The change-over from one type of reaction to the other merits some consideration. Sometimes this change is very rapid, but in other cases, more especially when the complement fixing antibody is changing back to inhibiting, there is a brief or more prolonged period when a marked prozone is shown in the complement fixation test and inhibition as well as fixation is found. The results of the few experiments using mixed sera throw some light on this problem, but by no means solve it. It is clear that either antibody in gross excess can completely mask the action of the other, and it appears that the proportions necessary for the masking of one antibody vary considerably in different pairs of sera tested. Usually when inhibiting and fixing antibodies are present in similar amounts the prozone phenomenon is seen in the complement fixation reaction, while inhibition can also be demonstrated. The slow change from fixation to inhibition which occurs during the fading antibody response may well be due simply to the presence of the two types of antibody.

The exceedingly rapid change-over sometimes seen in the secondary response is more difficult to explain. Even assuming that the whole of the antibody production changes from inhibiting to fixing type in a single day, the complete change from one type of reaction to the other in seven days or less, when each is found in high titre, is surprising. If the half-life of pigeon antibody is taken as similar to the 6·8 days given by Wood *et al.* (1953) for Lansing antibody in rhesus monkeys, then in such cases the fixing antibody would be unlikely to be in greater excess than 2:1 on the first occasion when fixation only was seen. The results of the experiments done so far on mixed sera suggest that complete dominance of fixing antibody in such a mixture might be possible.

The reactions of the various species of birds which have been tested by different workers are shown in Table 5. There is agreement that fowl sera

do not normally fix complement although, in the work of Karrer, Meyer and Eddie and in that reported here, occasional low titre complement fixing sera have been found. Turkeys, ducks and geese have not been shown to fix complement. Most other species which have been tested at all have only been tested by the direct complement fixation test and it is clear that some birds of many species are positive by this test.

TABLE 5.

Type of reaction given by sera of birds of different species in the complement fixation test.

Species of bird	Complement fixation test	Complement fixation inhibition
Pigeon	Some birds 2,3	Some birds always 2,3 All birds at certain stages of immunization 3
Indian Dove *(Streptopelia suratensis)*	Some birds 3	Some birds always All birds at certain stages of immunization 3
Fowl	Very occasional and to low titre 2,3	All birds 1,2,3
Turkey	Never recorded	All birds 1
Duck	Never recorded	All birds 1,2,3
Goose	Never recorded	All birds 1
Records of single birds tested by both tests in this series.		
White-backed magpie *(Gymnorhina hypoleuca)*	Negative	Positive
Native hen *(Tribonyx ventralis)*	Doubtful	Positive
Swamp hen *(Porphyrio melanotus)*	Negative	Positive
Little black cormorant *(Phalocrocorax ater)*	Negative	Positive
Darter *(Anhinga novae-hollandiae)*	Negative	Positive

1. Rice (1948). 2. Karrer, Meyer and Eddie (1950). 3. Miles.

It would appear that the inhibiting antibody lacks some character which would enable it to combine with guinea-pig complement. This lack is clearly not the same as that in the incomplete antibody of Coombs, Mourant and Race (1945) since these bird sera can give agglutination in the presence of a suitable antigen. It is not known whether they would be able to give complement fixation with homologous complement as can horse sera (Hole and Coombs, 1947), which were shown by Rice to give inhibition in the presence of guinea-pig complement. Fowl complement is very unsatisfactory to work with and a suitable species is needed for investigating this problem.

The results reported here together with those of other workers suggest that there may be a general pattern of reaction among birds in the complement fixa-

tion reaction. It seems likely that in all birds the first response, and perhaps the sole response to a minimal stimulus, is the production of inhibiting antibody. Later and with a large stimulus a varying proportion of certain groups of bird produce an antibody capable both of combining with antigen and fixing guinea-pig complement. With greater intensity and with prolongation of the stimulus, more birds will give a positive complement fixation tests. Some species normally produce only inhibiting antibody, but with very intense stimulation a few individuals in such species may also give some complement fixation.

As the stimulus diminishes all birds will revert to producing the inhibiting type of antibody before all antibody demonstrable by either the direct or the indirect test finally disappears.

Conclusion.

As a result of these experiments it is suggested that it is likely that in complement fixing systems bird sera will first after infection produce complement fixation inhibiting antibodies. In some species and probably in some of the main groups of birds (for instance, the *Galliformes*) this will normally be found to be the only type of reaction given. In other species and groups (as in this case the *Columbiformes*) a varying number of individuals may, if the stimulus is adequate, produce complement fixing antibodies.

As the stimulus to antibody production fades the complement fixing antibody will be replaced by inhibiting antibody, before the final disappearance of all reaction.

SUMMARY.

During the course of immunization of homing pigeons and Indian doves (*Streptopelia suratensis*) with Murray Valley encephalitis virus, in all birds first of all complement fixation inhibiting antibodies were formed. In a variable proportion of birds, the inhibiting antibody later gave way to antibody capable of fixing guinea-pig complement. As the antibody faded, the fixing antibody in these birds was again replaced by inhibiting antibody before all antibody finally disappeared. The more intense the antigenic stimulus the higher the proportion of birds which produced complement fixing antibody at some stage of the disease.

Among domestic fowls tested, one bird gave a very low positive complement fixation as well as inhibition, domestic ducks and a number of species of native Australian birds were found to give inhibition, but inadequate numbers were tested to show whether fixation ever occurred in these species.

It was suggested that, in birds, a general pattern of reaction occurred with

an antigenic stimulus first causing inhibiting antibody. This, in certain individuals of some species given adequate stimulation, would later be replaced by complement fixing antibody. The more powerful the stimulus, the more individuals and the greater the number of species in which fixation would be found.

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INFECTION OF BIRDS WITH MURRAY VALLEY ENCEPHALITIS (X DISEASE)

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When the outbreak of encephalitis occurred in the Murray Valley in 1951 and a virus belonging to the Japanese B group was isolated, the possibility that this disease also was likely to be primarily one of wild birds occurred to all workers interested in the virus. Anderson *et al.* (1952) showed antibodies in a variety of wild birds and domestic fowls, Miles (1952) showed susceptibility of certain species of birds to infection in the laboratory. Miles and Howes (1953) reported finding positive sera among wild water birds along the South Australian section of the Murray, and Anderson (1953) gave the detailed findings on the birds which he had collected and also showed that sera from newly hatched little pied cormorants *(Phalocrocorax melanoleucos)* might contain neutralizing antibody to the virus which could be demonstrated for up to 15 weeks after hatching, but had disappeared by the time the birds were six months old.

In this paper more detailed laboratory studies on the infection in a number of species of bird and on the course of antibody production and disappearance are recorded.

MATERIALS AND METHODS.

The main birds used in these experiments were domestic fowls, homing pigeons and Indian doves *(Streptopelia suratensis)*. Two domestic ducks, two silver gulls *(Larus novae-hollandiae)* and one white-backed magpie *(Gymnorhina hypoleuca)* were also used.

In the early experiments, which were designed primarily to discover whether the birds were susceptible to the infection, large doses of virus were inoculated either intracerebrally, intramuscularly or intradermally. In later experiments doses thought to be of the order of size which a mosquito could inject were inoculated intradermally except in certain experiments on Indian doves.

In the earlier experiments blood from birds either singly or after pooling two or three bloods was tested by the inoculation of undiluted heparinized blood in 0·03 ml. volumes intracerebrally into weaned mice.

The results obtained by this crude technique suggested that there were substantial variations in the amount of virus in the blood as well as in the duration of viraemia and in

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TABLE 1.

Viraemia in Indian doves (Streptopelia suratensis).

Dove	Dose of virus in mouse LD_{50}	Route of inoculation	Deaths of mice inoculated intracerebrally on days after inoculation of bird. 6 hrs.	1	2	3	4	5	6	7	8	9
2	10^8	I.D.	0/2*,†	100% 1/2 10^{-2} 1/2 10^{-4} 0/2	100% 2/2 10^{-2} 0/2 10^{-4} 0/2	100% 2/2 10^{-2} 0/2 10^{-4} 0/2	100% 2/2 10^{-2} 1/2 10^{-4} 0/2	0†	N.T.‡	0	0	0
3	10^8	I.D.	2/2	100% 2/2 10^{-2} 2/2 10^{-4} 1/2	100% 2/2 10^{-2} 1/2 10^{-4} 0/2	100% 1/2 10^{-2} 0/2 10^{-4} 0/2	——— Dead—no virus detected in brain ———					
6	50	I.C.	0/2	100% 2/2 10^{-2} 0/2 10^{-4} 0/2	100% 2/2 10^{-2} 0/2 10^{-4} 0/2	100% 2/2 10^{-2} 0/2 10^{-4} 0/2	0	0				
7	50	I.C.	0/2	100% 2/2 10^{-2} 1/2 10^{-4} 0/2	100% 2/2 10^{-2} 2/2 10^{-4} 0/2	100% 2/2 10^{-2} 0/2 10^{-4} 0/2	0	0				
8	50	I.D.	0/2	100% 1/2 10^{-2} 0/2 10^{-4} 0/2	100% 2/2 10^{-2} 0/2 10^{-4} 0/2	100% 2/2 10^{-2} 2/2 10^{-4} 0/2	100% 1/2 10^{-1} 0/2 10^{-2} 0/2	100% 1/2 10^{-1} 0/2 10^{-2} 0/2				
9	50	I.D.	0/2	0	0	0	0	0				
1	50	I.D.	0/4	100% 1/2 10^{-2} 1/4 10^{-4} 0/4	100% 4/4 10^{-2} 0/4 10^{-4} 0/4	100% 2/4 10^{-2} 0/4 10^{-4} 0/4	100% 2/4 10^{-2} 0/4 10^{-4} 0/4	0	N.T.	0	0	0
4§	10	I.D.	0/2	0	0	0	0	0				
5§	10	I.D.	0/2	0	0	0	0	0				

* Numerator = number of mouse deaths, denominator = number of mice in group.

† Where denominator is shown when no infection was found only undiluted blood was tested; where dilutions were tested a negative is signified by 0.

‡ Not tested.

§ Serological evidence suggested that infection failed to occur.

later experiments the heparinized blood was titrated intracerebrally in weaned mice. The use of the embryonated hen's egg might have revealed even smaller amounts of virus than the mouse, but we have preferred to use the latter which ordinarily shows a very characteristic picture when infected with the virus, so that it is not normally necessary to use a second passage to confirm the presence of virus.

Complement fixation test. The preparation of the infected and the normal mouse brain antigens and the technique of the complement fixation test were those previously described (Miles and Howes, 1952).

Complement fixation inhibition test. The development of the complement fixation inhibition test used and the final technique adopted is described in the preceding paper (Miles, 1954).

Neutralization test. The technique used was that described in an earlier paper (Miles and Howes, 1952).

RESULTS.

Viraemia.

The results of the experiments on the viraemia in birds after infection with the virus are shown in Tables 1 to 4.

TABLE 2.

Viraemia in domestic fowls.

Fowl	Dose of virus in mouse LD_{50}	Route of inoculation	Deaths of mice inoculated intracerebrally on days after inoculation of bird						
			1	2	3	4	5	6	7
14-day chicks (pool of two bloods)	$10^{7 \cdot 7}$	I.C.	2/2*,†	2/2	1/2	1/2	0/2	0/2	0/2
14-day chicks (pool of two bloods)	$10^{7 \cdot 7}$	I.D.	Non-specific deaths	2/2	2/2	0/2	0/2	0/2	0/2
Cockerel 1	10^{9}	I.M.	0/4	0/4	0/4	0/4	0/4		
2	10^{9}	I.M.	0/4	4/4	2/4	0/4	0/4		
3	50	I.D.	0	0	0	0	0		
4‡	50	I.D.	0	0	0	0	0		
5	50	I.D.	0	0	0	0	0		
6‡	50	I.D.	0	0	0	0	0		

* Numerator = number of mouse deaths, denominator = number of mice in group.

† Where denominator is shown only undiluted blood was tested; where dilutions were tested a negative is signified by 0.

‡ Serological evidence suggested that infection failed to occur.

A small experiment was carried out in the Indian doves to give an indication of the effects of dosage and route of inoculation on viraemia (Table 1). Ten mouse LD_{50} failed to infect the two birds tested, but with doses of 50 mouse LD_{50} or greater there seemed to be little difference in the amount of virus in the blood or in the duration of viraemia, except that in one of the three birds inoculated intradermally with 50 mouse LD_{50} no viraemia was found although serology suggested that infection had occurred. One bird which had received 10^{8} mouse LD_{50} intradermally was found dead on the fourth morning having been appar-

ently well the night before. No virus could be detected in the brain of this bird and the cause of death is uncertain. One of the birds receiving 50 mouse LD_{50} intradermally showed viraemia lasting at least 5 days. The remainder which showed viraemia at all had virus in the blood for 3 or 4 days regardless of route or size of dose.

In the adult cockerels tested with 50 mouse LD_{50} two of four failed to give serological evidence of infection (Table 2). No viraemia was found in the other two at any time. Two birds were given a massive dose intramuscularly and one of these had virus in the blood on the second and third day after inoculation. No virus was demonstrated in the blood of the other at any time.

The results with 14-day-old chicks contrast strongly with those in the adult cockerels. With large doses of virus inoculated intracerebrally or intradermally viraemia was present up to the third or fourth day after inoculation, but not later. Some of these birds died between 3–4 weeks after inoculation but virus was not recovered from the brains of either group. The death may well have been due to the inadequacies of the improvised brooder in which they were kept.

Viraemia in homing pigeons was transient and in four birds tested after inoculation with 50 mouse LD_{50} intradermally only lasted one to two days, although in one bird virus was detected in blood diluted to 10^{-4} 48 hours after inoculation (Table 3). One of the two further birds inoculated with the same dose, but not investigated for viraemia, failed to give serological evidence of infection.

TABLE 3.

Viraemia in homing pigeons.

Pigeon	Dose of virus in mouse LD_{50}	Route of inoculation	Deaths of mice inoculated intracerebrally on days after inoculation of bird								
			6 hrs.	1	2	3	4	5	6	9	12
1–3 pooled	10^8	I.C.	N.T.*	N.T.	N.T.	4/4†‡	N.T.	N.T.	0/4	0/4	0/4
4–6 pooled	10^8	I.D.	N.T.	N.T.	N.T.	0/4	N.T.	N.T.	0/4	0/4	0/4
7	50	I.D.	0/2	0§	0	100% 2/2 10^{-2} 0/2 10^{-4} 0/2	0	0			
8	50	I.D.	0/2	0	100% 2/2 10^{-2} 2/2 10^{-4} 1/2	100% 2/2 10^{-2} 1/2 10^{-4} 0/2	0	0			
9	50	I.D.	0/2	0	0	100% 0/2 10^{-2} 1/2 10^{-4} 0/2	0	0			
10	50	I.D.	0/2	0	100% 1/2 10^{-2} 0/2 10^{-4} 0/2	0	0	0			

* Not tested.

† Numerator = number of mouse deaths, denominator = number of mice in group.

‡ Where numerator and denominator are shown but no blood dilution, only undiluted blood was tested.

§ Where dilutions of blood were tested a negative is signified by 0.

TABLE 4.

Viraemia in miscellaneous birds.

Bird	Dose of virus in mouse LD_{50}	Route of inoculation	Deaths of mice inoculated intracerebrally on days after inoculation of birds. 6 hrs.	1	2	3	4	5	6	7	8	10
Duck 1	10^7	I.C.	0/2*,†	4/4	4/4	3/4	1/4	0/4	0/4	0/4	0/4	0/4
Duck 2	10^7	I.D.	0/4	4/4	4/4	4/4	Dead					
Silver gull 1 *(Larus novae-hollandiae)*	10^8	I.C.	N.T.‡	4/4	10^{-2} 4/4§ 10^{-3} 3/4 10^{-4} 1/4	4/4	4/4	N.T.	4/4	N.T.	2/4 Died	
Silver gull 2	10^8	I.C.	N.T.	4/4	10^{-2} 4/4§ 10^{-3} 4/4 10^{-4} 2/4	4/4	4/4	N.T.	3/4	Died—virus present in brain		
Magpie *(Gymnorhina hypoleuca)*	10^8	I.D.	4/4	10^{-2} 4/4 10^{-4} 3/4 10^{-6} 0/4	10^{-2} 4/4 10^{-4} 2/4 10^{-6} 0/4	10^{-1} 4/4 10^{-2} 4/4 10^{-3} 3/4	100% 4/4 10^{-1} 4/4 10^{-2} 0/4	100% 2/4 10^{-1} 0/4 10^{-2} 0/4	100% 1/4 10^{-1} 0/4 10^{-2} 2/4	100% 1/4 10^{-1} 0/4 10^{-2} 0/4	0**	

* Numerator = number of mouse deaths, denominator = number of mice in group.

† Where numerator and denominator are shown but no blood dilution, only undiluted blood was tested.

‡ Not tested.

§ Blood was titrated after 3 months' storage in dry ice.

** Where dilutions of blood were tested a negative is signified by 0.

The remaining six birds received large doses of virus intracerebrally, but for the reasons given above it is thought that the viraemia found would not be greatly different even if the dose of virus were reduced.

Virus was not titrated in the blood of the two ducks, but the early death of the mice inoculated with undiluted blood indicated that substantial amounts were present (Table 4). Both ducks were sick on the third day after inoculation and one died on the fourth. The other recovered after showing viraemia for four days. The difference between these findings for adult ducks and our findings for cockerels may be of significance.

The two silver gulls tested were both birds captured while they had broken wings. They adapted themselves easily to laboratory life and after the broken distal fragment of the wing had been amputated, fed well. After intracerebral inoculation of virus both these birds began to appear ill, showing leg weakness from the third day after inoculation. One died on the sixth day and the other on the eighth day after inoculation (Table 4). Virus was isolated from the brain of the bird dying on the sixth day, the other was not tested. Viraemia continued throughout the life of these birds and 48 hours after inoculation death occurred in mice inoculated with blood diluted to 10^{-4}. The one white-backed magpie had a viraemia lasting seven days and blood taken 24 and 48 hours after inoculation diluted to 10^{-4}, killed mice (Table 4).

There were few cases in which diluted blood showed active virus when undiluted was inactive. The blood of the magpie on the sixth day after inoculation gave one death among four mice undiluted, none at 10^{-1} and two at 10^{-2} and one pigeon was positive at 10^{-2} but not undiluted on the third day.

Serology.

The most detailed study of the serology of MVE infection in birds was carried out in pigeons. In these birds, whether complement fixing antibodies appeared or not, complement fixation inhibiting antibodies developed earlier and were first found from the 6th to the 14th day, reaching a maximum between the 10th and 20th day. Complement fixing antibodies replaced the inhibiting antibodies in three of the seven pigeons observed closely after a single inoculation of virus. Complement fixation was found in one bird on the 14th and 18th days only, in another from the 10th to the 42nd day, while in a third partial fixation was observed on the 42nd, 56th and 76th day, inhibition also being present. The full details of this work are given in the previous paper. Among six other pigeons tested 35 days after inoculation only three gave positive complement fixation and three complement fixation inhibition.

In all birds adequately tested the neutralization test became positive later than the complement fixation inhibition test and the results suggested that a maximum titre was usually reached between one to two months after infection. On two occasions low titre neutralization was found on the 14th day after the

inoculation of a large dose of virus to pigeons, but in five other pigeons and in two cockerels no significant amount of neutralizing antibody could be demonstrated at the same time.

Table 5 shows the maximum titre and duration of inhibiting antibody and of neutralizing antibody in those birds sufficiently frequently tested for the results to be of value. It will be seen that in many cases the complement fixation inhibition antibody was only present for a brief period although in the duck and the magpie a good titre was maintained for at least a year. Neutralizing antibody was much more enduring and in every case demonstrable titres of neutralizing antibody were present in the last specimen tested.

TABLE 5.

Bird	Duration of viraemia (days)	Max. titre C.F.†	Duration of C.F. antibody (days)	Max. titre N.1	Duration of neut. antibody (days)
Cockerel 1	0	1:80	>92	3·2	>92
2	2	1:80	>92	3·9	>92
3	0	1:320	>148	3·0	>148
5	0	1:160	92	3·1	>148
Indian dove 1	4	1:10*	99	3·5	>169
2	4	1:20*	99	>3·9	>151
6	3	>1:320	>162	>2·9	>127
7	3	1:80	>162	3·2	>127
8	5	1:80	71	3·0	>127
9	0	1:40	57	3·0	>127
Pigeon 7	2	1:40	56	2·5	>84
8	2	1:80	56	2·2	>84
9	1	1:20	42	1·4	>84
10	1	1:80	56	1·5	>84
Duck 1	4	1:80	>398	3·4	>398
Magpie	6	1:80	>344	2·8	>344

* No sera tested at the time when C.F. titre would be expected to be at its highest.
† Either by the direct test or by the inhibition (indirect) test.

No close relation could be demonstrated between the length and intensity of viraemia and the duration of the complement fixing inhibiting antibody in the small series of birds watched both for viraemia and for antibody production.

Antibodies in wild birds.

The complement fixation and complement fixation inhibition tests were applied to nine birds positive by the neutralization test, to three birds whose neutralization results had been regarded as doubtful and the 20 birds negative to the neutralization test. The results are shown in Table 6.

Four of the nine neutralizing positive sera gave a positive test also by the complement fixation inhibition test and one of these gave a doubtful lower titre by direct complement fixation. Of the three doubtful sera, all of which came from darters *(Anhinga novae-hollandiae)*, one gave a low titre positive inhibition test.

TABLE 6.

Bird	Neutralization	Complement fixation	Complement fixation inhibition
Black swan *(Cygnus atratus)*	+	0	0
Chestnut teal *(Querquedula castanea)*	+	0	0
Native hen *(Tribonyx ventralis)*	+	±1:10	1:20
" " " "	+	0	0
" " " "	+	N.T.	0
" " " "	+	N.T.	0
Eastern swamp hen *(Porphyrio melanotus)*	+	0	1:5
White-faced heron *(Notophoyx novae-hollandiae)*	+	N.T.	1:20
Little black cormorant *(Phalacrocorax ater)*	+	0	1:10
Darter *(Anhinga novae-hollandiae)*	±	0	1:10
" " "	±	0	0
" " "	±	0	0

The remaining 20 birds were negative to all three tests.

These sera were collected between four to ten months after the 1951 epidemic in the Murray Valley and sera collected earlier might have given higher titres and a better proportion of positives by the *in vitro* tests, but at the time at which they were taken there were twice as many positive results by the neutralization test. No serum which was unequivocally negative by the neutralization test was positive by the *in vitro* tests.

Feather lice.

Numerous *Mallophaga* were present on the two gulls investigated and these were collected at the time of death of the birds. Some were sent to Miss Theresa Clay of the British Museum, who identified them as an *Ostromenopon* species. The remainder after storage for three to five days were ground up as a 10 p.c. suspension and inoculated intracerebrally into weaned mice. No virus was isolated.

DISCUSSION.

Hammon, Reeves and Sather (1951a) have suggested that viraemia might be more prolonged and intense in birds infected with small doses than with large doses of Japanese B virus, because of the greater early antigenic stimulus which might be expected with the large dose. In the work reported here a limited attempt has been made to examine this theory and the results suggest that with the virus of Murray Valley encephalitis, provided that the dose of virus injected is sufficient to infect the birds, the dose and route of inoculation do not greatly affect the amount of virus in the blood or the duration of viraemia. It is likely that those species not tested would have behaved in the same way.

The adult cockerel showed viraemia less regularly than any other bird tested and appeared to be less easily infected with 50 mouse LD_{50} of virus than

Indian doves or pigeons. This finding is in line with that of Hammon *et al.* (1951 a and b) in which they found only minimal viraemia in fowls infected with small doses of Japanese B or St. Louis virus as against substantially greater viraemia in certain wild birds tested. Fourteen-day-old chicks showed much more viraemia than the cockerels and possibly their deaths were attributable to the disease. Of the other birds tested the susceptibility appeared to grade from the pigeons, which gave only a brief viraemia and showed only slight ill health after large doses of virus, to silver gulls, which showed prolonged viraemia and died while still viraemic.

The relatively prolonged viraemia found in the Indian doves, ducks, gulls and magpie as compared with the cockerels and pigeons would suggest that the former birds would provide a much better source for infecting mosquitoes than the latter, and since the epidemics occur at a time of year when very few young chicks are available, it seems likely that, despite their proximity to human habitation, domestic fowls are not of great significance in the spread of the disease. Domestic ducks, however, might be an important source of mosquito infection.

The serological results given in this paper show clearly that in any inter-epidemic survey of bird sera, if one test only is to be used, the neutralization test is the test of choice. During an epidemic this test would be likely to miss recent infections and then both complement fixation and complement fixation inhibition tests should also be included. In the present state of our knowledge of these tests it would be wise to use both tests on all specimens although the inhibition test will give far more positives than the direct test.

Howitt, Dodge, Bishop and Gorrie (1948) have reported the isolation of Eastern equine encephalomyelitis virus from chicken lice *(Eomenacanthus stramineus)*. The systematics of the *Mallophaga* are not very satisfactory, but I am given to understand that this genus is quite closely related to the *Ostromenopon* genus to which Miss Clay has assigned the lice from the gulls tested in this series. These biting lice did not contain virus although they had been six to eight days on birds with substantial viraemia. They appear only to take blood casually and occasionally and it was not thought that they were likely to be of any importance as vectors.

SUMMARY.

Indian doves *(Streptopelia suratensis)* inoculated with varying doses of virus showed no significant variation in viraemia provided sufficient virus was present to infect the bird.

Viraemia was not regularly demonstrated in adult cockerels, but was in 14-day-old chicks. Viraemia in pigeons only lasted 24–48 hours.

Indian doves, ducks, silver gulls *(Larus novae-hollandiae)* and a white-backed magpie *(Gymnorhina hypoleuca)* showed a more prolonged viraemia.

Complement fixation inhibiting antibodies developed early in all birds and in some complement fixing antibodies developed. Neutralizing antibodies appeared later, but were usually more enduring.

Re-examination of wild bird sera taken 4–10 months after the 1951 epidemic showed that five of nine sera positive and one of three doubtful by the neutralization test were positive by complement fixation inhibition. Twenty sera were negative to all tests.

Virus was not isolated from a pool of *Ostromenopon sp.* biting lice which had fed on gulls with viraemia for six or eight days.

Acknowledgments. I wish to thank Mr. Howes for preparing the antigens used in these experiments and Miss Theresa Clay for identifying the *Mallophaga.*

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THE INFLUENCE OF TAB VACCINE ON THE TOXICITY OF HISTAMINE AND RELATED PHENOMENA

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In view of the findings of the effect of soluble antigen (diphtheria toxoid, Trethewie and Gaffney, 1951), on the histamine content of lungs and the toxicity of histamine, it was thought worthwhile to determine if particulate antigen (TAB vaccine) affected these estimates also. Further, in view of the findings of Miklos Jansco (1947) concerning the effect of histamine on reticulo-endothelial cell activity, it was considered advisable to investigate the effect of TAB vaccine on the ability of the liver to remove perfused particulate matter.

METHOD.

TAB inoculation.

Fortnight-old guinea-pigs were randomized, numbered and weighed. Control animals were injected with phenol tricresol saline (TAB diluent) 0·1 ml. weekly subcutaneously for 7 weeks, and the experimental animals with 0·1 ml. TAB (Commonwealth Serum Laboratories).

Histamine content of lungs.

The lungs were perfused free of blood and dried firmly between filter paper, weighed and ground with sand in Tyrode and boiled.

The histamine was assayed on the isolated jejunum of the guinea-pig as histamine acid phosphate. Feldberg and Kellaway (1937 a, b) have shown that the substance from the lung which causes immediate contraction of the gut is histamine and Trethewie (1938) has shown that this yields the same figure as digestion with acid (Best, 1929).

Liver perfusion.

The isolated liver (guinea-pig) was perfused through the portal vein with Tyrode at 3–5 ml./min. (T 37° C.) and after the fluid issuing from the liver became free of blood, a sample was collected (S_1). Then an Indian ink suspension in Tyrode which was kept agitated was perfused through the liver *via* a Y piece and narrow tubing attached proximally to the portal vein and the originally connected Tyrode solution was clipped off. The Indian ink perfusion fluid (100 ml.) was used to completion (3–5 ml./min.) and the whole sample collected (S_2). Control samples of ink suspension (C) were collected from the tubing immediately proximal to the portal vein at the beginning and end of the ink perfusion. The Indian ink perfusion fluid, which was made up freshly every three days, was made up as follows: 8 drops of Indian ink (Reeves' fixed, obtained after shaking the bottle) were added from a pipette to 100 ml. Tyrode. Then 10 ml. of this solution was added to 90 ml. Tyrode for "non-histamine" perfusions and to 80 ml. Tyrode + 10 ml. histamine (1 in 2 million Tyrode) for the "histamine"

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perfusions. The sample obtained after ink perfusion (S_2) was assayed for loss of particulate matter on a Turbidimeter against the earlier sample collected (S_1) as 100 (for S_1 was occasionally a little turbid). The control sample (C) was also assayed against Tyrode as 100. The actual amount of particulate matter taken up was estimated as loss of optical density of perfusate (S_2) $\times$ 1,000 divided by the weight of the animal in grams divided by the optical density of the standard (C).

EXPERIMENTAL.

Phagocytic activity of the Kupffer cells of the liver.

When guinea-pigs were inoculated with TAB vaccine (0·1 ml.) weekly for 7 weeks and the liver perfused intraportally one week after the final injection, there was no evident change in the phagocytic activity of the liver. The figures for the amount of particulate matter taken up were 1·21, 1·38, 1·55, 1·92, 2·07 and 2·50 for the "histamine" perfusions and 2·21 for the "non-histamine" perfusion (Table 1). These figures were estimated as described in the method. This corrects for the difference in size of animal (a larger liver can be expected to take up more ink) and differences in the amount of ink used owing to slight sedimentation. This variation is considerably less than the variation in histamine content of the corresponding lung. The amounts of particulate matter taken up by the control livers similarly estimated were 0·99, 1·61, 1·86, 2·06 and 2·44 for the "histamine" perfusions and 1·75, 1·88 and 2·16 for the "non-histamine" perfusions (Table 1). These findings are also recorded in Fig. 1 where the open columns indicate the absorption for the control histamine perfusions and the opaque columns indicate the absorption for the experimental ("TAB injected") histamine perfusions. The average absorption for the former was 1·77 and for the latter 1·79. Including the "non-histamine" perfusions the figures become 1·83 and 1·84 respectively.

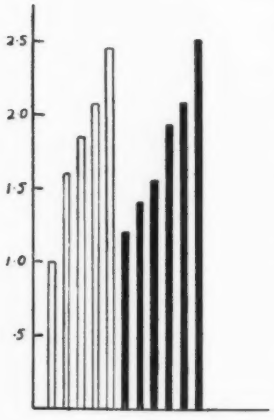

Fig. 1. Ordinate: Uptake of particulate matter. Open columns control, opaque columns experimental perfusion. Details in text.

Therefore there is no evident difference in the phagocytic activity of the liver for inert particles after treatment with TAB vaccine.

Effect of repeated TAB inoculation on the histamine content of the lungs of guinea-pigs.

When guinea-pigs were inoculated with TAB (0·1 ml.) weekly for 7 weeks, 1 week after the final injection the lung content became on the average 44·6 μg. H/gm. compared with an average of 38·1 μg. for the uninoculated. This rise

(14 p.c.) is not significant statistically. It is in marked contrast to the increase of 50 p.c. in animals treated with diphtheria toxoid (Trethewie and Gaffney, 1951). When the total lung histamine (content × weight) is compared the difference is greater, 77·8 μg. compared with 109·7 μg. for the injected, but this becomes reduced again to 19 p.c. when the total content is divided by the total weight of animal (0·26 μg./gm. animal for control and 0·31 for the injected). These findings are recorded in Table 2. It is evident that TAB vaccine does not produce the marked increase in histamine content noted with peptone, hormone, or toxoid.

TABLE 1.

Density of particulate matter absorbed × 1,000 (hepatic) in control and TAB-injected animals—per gm. animal per unit particle injected.

	Treated	Control
	1·38	2·44
	2·50	0·99
	1·92	1·86
	1·55	2·06
	1·21	1·61
	2·07	
Average	1·77	1·79
Absorption without added histamine in the perfusion fluid.		
	2·21	1·75
		1·88
		2·16
Average	1·83	1·84

TABLE 2.

Histamine content of the lungs of guinea-pigs, control, and after weekly injection of TAB vaccine (treated).

No.	Histamine μg./gm.	Total histamine control	μg./g.a.
1	167	367·4	1·16
2	20	38	0·12
3	7	20·3	0·06
4	30	99	0·22
5	11	31·9	0·06
6	25	57·5	0·15
7	20	30	0·11
8	25	62·5	0·21
Average	38·1	77·8	0·26
		Treated	
1	155	356·5	0·95
2	40	104	0·22
3	19	43·7	0·12
4	40	84	0·28
5	20	44	0·12
6	10	32	0·08
7	28	103·6	0·40
Average	44·6	109·7	0·31

Effect of TAB inoculation on the toxicity of histamine.

While diphtheria toxoid reduced greatly the toxicity of histamine for a guinea-pig we were not able to detect a difference with animals treated with TAB vaccine. Animals were injected weekly for 7 weeks and then 7 weeks later tested for the toxicity of histamine.

The LD_{50} (Probit) for the treated was 10 mg./kg. compared with 9·8 mg./kg. for controls. These animals were aged 4 months. These findings are recorded below (Table 3).

TABLE 3.

Toxicity of histamine for control and treated (TAB-injected) guinea-pigs.

Dose of histamine subcutaneously mg./kg.	Treated			Control			
5·5				S 368	S 370	S 450	
8·25	S 420	S 400	S 450	+ 390	+ 430	S 440	S 440
10	+ 510	+ 530	S 450		S 540		
11·55	+ 400	+ 450					
12	+ 450	+ 460	S 540	+ 390	+ 370	S 390	

+ Death; S Survival.

DISCUSSION.

In efforts to determine fundamental aspects of a problem we have termed "non-specific" immunity (Trethewie, 1949) variation in the toxicity of histamine, the ease of its release and its level in the lungs has been determined in relation to ageing, peptone administration, endocrine and toxoid administration. In general it has been found that after a very high initial content at birth which rapidly falls, simple ageing is associated with an increase in the histamine content of the lung (Trethewie, 1947). Ageing is also associated with an increase in the lethal dose of histamine and histamine becomes less readily released by tissue injury (Trethewie and Day, 1949). Peptone affects the histamine content similarly but makes histamine more readily releasable by another injurious agent (trypsin) (Trethewie and Day, 1948). Also the lethal dose of histamine is increased. Similar effects were obtained with gonadotropin and sex hormone (Trethewie and Gaffney, 1949, 1950) and diphtheria toxoid (Trethewie and Gaffney, 1951). On the other hand in the paper here reported we were unable to detect any marked difference in the histamine content of the lungs following TAB inoculation and the lethal dose of histamine was unchanged. It may be that TAB, derived from a bacterium affecting the "lymphocytic" series

of cells, affects a different cellular group than the other agents, and this may account for the difference. On the other hand it may be that TAB is simply far less effective as an antigen (say compared with diphtheria toxoid). It is to be remembered there is a general difference between the effectiveness of anti-toxic sera compared with anti-bacterial sera, i.e. the latter are not powerful agents and this may be due to a fundamental difference related to our findings.

Oakley has shown that TAB inoculated into the leg of a guinea-pig produces a local rise in antibody in the regional lymph node on the same side and not to any extent in the contralateral lymph nodes. The lymph node contains cells of the "lymphocytic" series which are the type of cells producing a response to typhoid infection. It is possible the immunological effect of TAB inoculation is produced *via* the lymphocyte from reaction between the bacterium and the lymphocyte.

This distinction, then, between diphtheria toxoid (marked histaminic effects — lung content, LD_{50}) and TAB (negligible histaminic effects) may be related to the difference in cellular response. Further in this regard it is to be remembered that Feldberg and Kellaway (1938) showed that staphylococcal toxin releases histamine from the liver but that the content of histamine increased on boiling. Microscopic examination of the centrifuged sediment which they showed me contained cells which appeared like reticulo-endothelium. On a volume basis mathematical deduction indicated that the histamine content of the cells was far greater than that of ordinary liver parenchyma. Though we are not justified in drawing too many conclusions it may be that the reticulo-endothelial cells are those which contain more histamine in the liver than ordinary parenchyma. It might be that toxin (Staphylococcal exo-toxin) here is acting on histamine containing cells and diphtheria toxoid might act similarly. TAB vaccine on the other hand, being bacterial body, might not act on them in the same way.

Further, we have shown that there is slight if any effect of TAB vaccine on the phagocytic activity of liver cells. We expected, if the reticulo-endothelial cells played a major rôle in phagocytic activity, that repeated injections of TAB might affect the activity of these cells in relation to another particle (Indian ink) in much the same way as we found with regard to histamine release by trypsin in relation to repeated peptone injections.

Burnet and Fenner point out that we may have two types of mechanism involved in antibody production. TAB belongs to one group and toxoid to the other. They consider that the macrophage does not produce antibody but will hand on materials to a nurse cell which forms antibody. Burnet and Fenner agree with the view that the plasma cell is the predominant antibody producer. It was concluded by them that there is enough evidence showing that bacterial agglutinin production in the rabbit differs very sharply from antitoxin production. The fowl responds well to particulate antigens yet with almost no response to diphtheria toxoid. Evidently the toxoid antibody production cannot

be interpreted in the manner of particulate antigen (Burnet and Fenner, 1949). We similarly showed a sharp distinction between a soluble agent producing marked histaminic effects while particulate agent is without any such effect.

It is worthy of notice that recently Pittman (1951) has shown that experimental whooping cough infection in mice is associated with a reduced lethal dose of histamine, but it is to be remembered that the testing dose was made while the animal was still recently sick and there are considerable lung lesions present at this stage. Histamine kills partly by its pulmonary effects so that the LD_{50} change may be partly non-specific. Further, the injection of histamine is made at a stage of infection when it may already have released some histamine into the circulation.

SUMMARY.

Repeated TAB inoculations have no statistically significant effect on the phagocytosis of ink in the liver (reticulo-endothelial cell activity) of the guinea-pig.

Repeated TAB inoculations had no significant effect on the histamine content of the lung of guinea-pigs or on the lethal dose of histamine.

The significance of the above findings in sharp contrast with the effect of soluble substances is discussed and special reference is made to immunological implications.

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DEAMINATION OF ASPARTIC ACID BY *PROTEUS* X-19

III. EFFECT OF K^+, Na^+ AND pH ON DEAMINATION BY WHOLE CELLS

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During an investigation into the effects of inhibitory materials on aspartic acid deamination by *Proteus* X-19, it was noted that dipicrylamine produces a marked depression of ammonia output. Since dipicrylamine is notable for its ability to combine with potassium ions, a study of the effect of the latter on the deamination reaction was carried out. It was found that not only potassium ions, but also sodium ions are necessary for the deamination of aspartic acid by whole *Proteus* cells. These requirements had not been detected previously, since the medium used in the investigation of aspartic acid deamination contained both metals in optimal amounts (Trudinger, 1951).

Materials and Methods.

The organism and general procedure have been described in an earlier paper (Trudinger, 1951).

The abbreviated description of buffers used in this paper is as follows:

K:Na	:	$KH_2PO_4 + Na_2HPO_4$
K:K	:	$KH_2PO_4 + K_2HPO_4$
Na:Na	:	$NaH_2PO_4 + Na_2HPO_4$
Na borate	:	Boric acid + NaOH + NaCl
K borate	:	Boric acid + KOH + KCl

In experiments on the influence of pH on the reaction rate, KCl or NaCl was added to the respective buffer solutions so that the K^+ or Na^+ concentrations remained the same over the complete pH range.

Neutralization of substrate, etc., was carried out with the metal hydroxide corresponding to the buffer. pH determinations were carried out on a Jones Model B electrometer. Sodium and potassium were determined on the Beckmann Model DU spectrophotometer with flame attachment (Wynn *et al.*, 1950). Fumarate was estimated by the method of Nossal (1952), succinate by the method of Krebs (1937). Anaerobic manometric experiments were carried out under nitrogen, with yellow P in the centre well. CO_2 was measured by the "direct method," bound CO_2 being released by 0·1 ml. of 5 N H_2SO_4 (Umbreit *et al.*, 1949).

All concentrations reported in this paper are final concentrations. Unless otherwise stated, the volume of reactants was 3 ml. and the reactions were carried out for 1 hour at 38° C.

RESULTS.

Effect of dipicrylamine.

The effect of dipicrylamine on aspartic acid deamination is shown in Table 1. Complete inhibition of the reaction was produced by 10^{-4} M inhibitor. However, the action of dipicrylamine may not in fact be an immobilization of potassium. It can be seen from Table 1 that dipicrylamine was just as effective in inhibiting the reaction in toluene-treated cells, in which no requirement for K^+ can be shown (Trudinger, 1953).

TABLE 1.

Effect of dipicrylamine on aspartic acid deamination.

0·0025 M L-aspartate; 0·066 M K: Na phosphate, pH 7·2; 0·3–0·6 mg. dry wt. cells (1–4 untreated, 5 toluene-treated).

Concentration of dipicrylamine	Q_{NH_3} 1	2	3	4	5
0	103	256	266	225	123
10^{-4} M	5	5	8	8	8
10^{-5} M	35	197	210	94	112
10^{-6} M				217	

1. Anaerobic, no additions. 2. Anaerobic + 10^{-3} M glucose. 3. Anaerobic + 10^{-3} M adenosine. 4. Aerobic, no additions. 5. Aerobic, 2 × 10^{-3} M $MgCl_2$.

Effect of potassium.

The deamination of aspartic acid in sodium phosphate buffer was strongly stimulated by the addition of KCl (Table 2). The reaction rate at pH 7·0 was almost optimal with 10^{-2} M KCl, but small additional increases in activity were observed as the KCl concentration was raised to 10^{-1} M (Fig. 4). Potassium sulphate was slightly less effective than KCl, due to slight inhibition by the sulphate ion. Rubidium salts were as effective as potassium; NaCl and Li_2SO_4 were inert. A small stimulation by CsCl may have been due to contamination by K^+ or Ru^+. K^+ in the form of phosphate, borate and acetate was as effective as KCl; potassium ions are therefore the active material.

TABLE 2.

Effect of KCl on deamination in sodium phosphate.

0·0025 M L-aspartate; 0·066 M Na-Na phosphate, pH 7·0; *ca.* 0·5 mg. dry wt. cells. Expt. 1, Anaerobic; Expt. 2, Aerobic.

Expt.	Additions	Q_{NH_3} No KCl	+ 10^{-2} M KCl
1.	None	28	100
	Glucose 10^{-3} M	135 (107)*	282 (182)
	Adenosine 10^{-3} M	143 (115)	300 (200)
2.	None	126	249

* Figures in brackets are the increases in activity produced by glucose or adenosine.

Stimulation by K^+ was observed in the presence or absence of glucose or adenosine (Table 2). On a percentage basis, the effect of K^+ on the "basic" activity was greater, stimulation being 3- to 4-fold compared with *ca.* 2-fold in the presence of glucose or adenosine. However, the activation of deamination was further enhanced by K^+.

Effect of sodium.

In view of the marked effect of K^+ on deamination rate, it was thought that a pure potassium buffer would be preferable to the mixed sodium and potassium usually employed. Under these conditions, however, low activities were again observed which responded markedly to the addition of NaCl (Table 3). Other members of the group I cationic series were ineffective. Na^+ was active when added as chloride, sulphate, phosphate and acetate.

TABLE 3.

Effect of NaCl on deamination in potassium phosphate.

0·0025 M L-aspartate; 0·066 M K:K phosphate, pH 7·0*; *ca.* 0·5 mg. dry wt. cells. Expt. 1, Anaerobic; Expt. 2, Aerobic.

Expt.	Additions	Q_{NH_3} No NaCl	Q_{NH_3} + 10^{-3} M NaCl
1.	None	55	181
	Glucose 10^{-3} M	143 (88)†	264 (83)
	Adenosine 10^{-3} M	145 (90)	272 (91)
2.	None	35	200

* Na^+ impurity in buffer was *ca.* 3×10^{-3} M.

† Figures in brackets are the increases in activity produced by glucose or adenosine.

In potassium buffer with added Na^+, the basic activity was much higher than that normally found in the K-Na buffers which have been generally used. The concentration of Na^+ required for optimal activity at pH 7·0 was *ca.* 10^{-2} M (Fig. 2), but, in contrast with K^+, the activity decreased as the Na^+ concentration was increased above 2×10^{-2} M. Variation in basic activity is correlated with Na^+ concentration rather than with K:Na ratio (Table 4).

TABLE 4.

Effect of K:Na ratio on deamination activity.

0·0025 M L-aspartate; anaerobic.

Buffers (0·066 M, pH 7·0) made from mixtures of potassium and sodium phosphates to give appropriate K:Na ratios.

Additions	K:Na ⟶ / Molarity of Na ⟶	7:1 0·013	3:1 0·023	Q_{NH_3} 1:1 0·053	1:3 0·085	1:7 0·094
None		177	177	122	81	85
Glucose		238	235	221	223	210
Adenosine		236	266	221	231	220
None*		205	205	198	204	202

* Aerobic.

The actual increase in QNH_3 produced by glucose and adenosine was not affected by the addition of Na^+ (Table 3). Furthermore, the activity in their presence was not significantly altered by concentrations of Na^+ sufficient to decrease the basic activity by up to 50 p.c. (Table 4).

Effect of pH.

It has been reported (Trudinger, 1951) that the pH for optimal activity of whole cells is between 6·8 and 7·2. As this curve was obtained using the usual Sörensen buffers made from KH_2PO_4 and Na_2HPO_4 (see Kolthoff and Rosenblum, 1937), the variation in K^+ and Na^+ concentration at different pH levels gives a distorted picture of the effect of hydrogen ion concentration. The effect of pH on deaminase activity was therefore re-investigated.

No potassium or sodium salts were available which were entirely free from contamination, and to offset any anomalies introduced by variation in this contamination, NaCl or KCl respectively was added to the appropriate buffer solutions so that the contaminating metal was present in equal concentration over the whole pH range. The level of impurity was checked in each case on the flame spectrophotometer. No significant concentration of metal was introduced with the cells.

The pH values of the various buffers were checked each day and substrate was neutralized to each respective pH. The change in pH during the reaction was never more than 0·1 and usually less than 0·05.

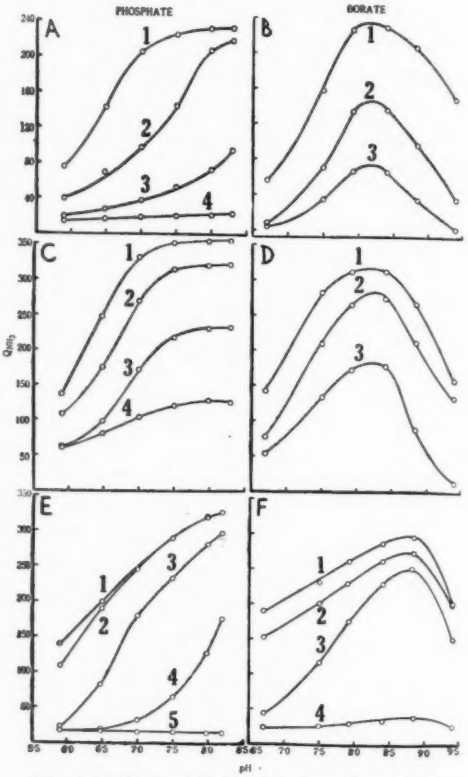

Fig. 1. Effect of Na^+ and pH on deamination in potassium buffer.

0·00375 M L-aspartate; buffers 0·066 M (K^+ = 0·133 M); A and B, anaerobic; C and D, anaerobic $+10^{-3}$ M glucose; E and F, aerobic.

Na^+ concentrations ($\times 10^3$ M)

A and C: 1, 10·7; 2, 5·7; 3, 2·7; 4, 0·7
B and D: 1, 11·2; 2, 3·2; 3, 1·2
E: 1, 25·7; 2, 10·7; 3, 5·7; 4, 2·7; 5, 0·7
F: 1, 11·2; 2, 6·2; 3, 3·2; 4, 1·2

When working at a pH above 7·5, small amounts of ammonia were expelled from the reaction medium. At pH 8·5, this loss was often as high as 50 p.c. This was overcome by using double side-armed tubes with substrate in one side-arm and in the other 0·1 ml. 5 N H_2SO_4 to fix NH_3. At the end of the reaction period, the acid was tipped into the main compartment, bringing the pH down to *ca.* 1–2 at which value aspartase is no longer active. The tubes were then shaken for a further 10 minutes to ensure complete absorption of any free ammonia in the tube. Under these conditions, 100 p.c. recovery of ammonia was obtained at high pH levels even when the reaction was run *in vacuo*.

The relation between activity and pH was found to depend on the ionic composition of the medium.

Experiments in potassium buffer.

The pH range for optimal anaerobic activity in potassium borate containing about 0·01 M Na^+ was between pH 7·9 to 8·4, both in the presence and absence of glucose (Fig. 1, B and D). In phosphate, high activity was obtained over a much wider pH range, the rate of deamination being almost optimal from pH 7·5 upwards (Fig. 1, A and C). Aerobically, the optimum was nearer pH 8·7 both in phosphate and borate (Fig. 1, E and F).

With lower Na^+ concentrations, the activity fell more rapidly as the pH was decreased from the optimum. This is due to a greater Na^+ requirement under the more acid conditions (Fig. 2). Thus anaerobically, optimal activity was found with 10^{-2} M Na^+ at pH 7·95, while at pH 5·9 the rate was still not maximal at $2{\cdot}5 \times 10^{-2}$ M Na^+; aerobically, the system required for optimal activity, 2–3 times as much Na^+ at pH 5·9 as at pH 8·5. Concentrations of Na^+ between $2{-}5 \times 10^{-3}$ stimulated markedly at the alkaline pH values, but not under acidic conditions.

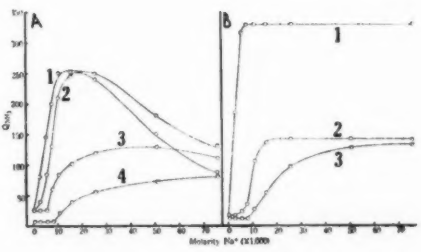

Fig. 2. Effect of Na^+ concentration on deamination.

0·00375 M L-aspartate; 0·066 M K: K phosphate.

A: Anaerobic, 1, pH 7·95; 2, pH 7·0; 3, pH 5·9; 4, pH 5·35.

B: Aerobic, 1, pH 7·95; 2, pH 5·9; 3, pH 5·35.

About 5×10^{-2} M Na^+ was required at pH 5·35. The inhibition of the basic anaerobic deamination by high concentrations of Na^+ also became less as the pH was lowered; at pH 5·35, no inhibition was observed with 10^{-1} M Na^+. A less pronounced increase in Na^+ requirement was also found at pH values above the optimal. Higher Na^+ concentrations were required for optimal activity, but deamination was increased significantly at Na^+ levels which had

no effect at acidic pH values. No work has been done at pH values above 9·5. Results obtained anaerobically were essentially similar when glucose was replaced by adenosine.

Experiments in sodium buffer.

In sodium buffers, two anaerobic pH optima were found in the absence of glucose (Fig. 3, A and B); a relatively weak optimum appeared at pH 6·4–6·5 and a stronger one between pH 7·8 and 8·2. These peaks occurred regardless of the K^+ concentration of the buffer. On the addition of glucose and 10^{-2} M K^+, a broad optimum range, from pH 7·0 to 8·0 in phosphate and from 6·5 to 7·5 in borate, was observed. At 10^{-5} M K^+, however, two peaks were observed in borate, the more acidic of which disappeared with increasing K^+ concentration. In phosphate, a flattening of the pH curve was the only evidence of a second pH optimum. In borate, increases in K^+ concentration to 10^{-2} M tended to lower the pH optimum.

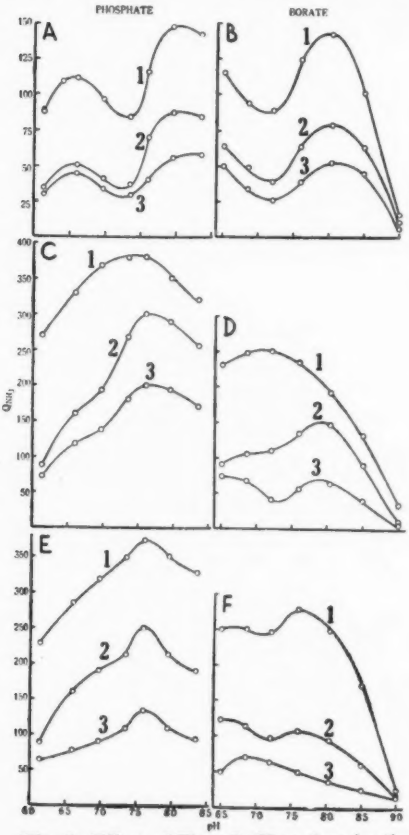

Fig. 3. Effect of K^+ and pH on deamination in sodium buffer.

0·00375 M L-aspartate; buffers, 0·066 M (Na^+ = 0·133 M). A and B, anaerobic, C and D, anaerobic + 10^{-3} M glucose; E and F, aerobic.

K^+ concentrations: Curves 1, 10^{-2} M; 2, 10^{-4} M; 3, 10^{-5} M.

Two aerobic pH optima were also evident in borate in the presence of low concentrations of K^+ (Fig. 3, F); as in the case of glucose-stimulated anaerobic activity, increasing K^+ to 10^{-2} M eliminated the more acidic peak. In phosphate, there was no well-defined aerobic pH optimum at the lower pH values; the curves were characterized by small but very sharp peaks between pH 7·35 and 7·95.

As in the case of sodium, low concentrations of K^+ had less pronounced stimulatory effects with decreasing pH. The results were less striking than those obtained with sodium, and with the basic anaerobic system and aerobic system an increased K^+ requirement for maximal activity at acid pH values was not found (Fig. 4). The stimulated an-

aerobic deamination showed greater K^+ requirement for optimal activity at acid pH values.

The basic anaerobic deamination in potassium buffer with 2×10^{-2} M Na^+ followed a linear course to completion. In sodium buffer with added K^+, the activity fell with time over an hourly period (Fig. 5).

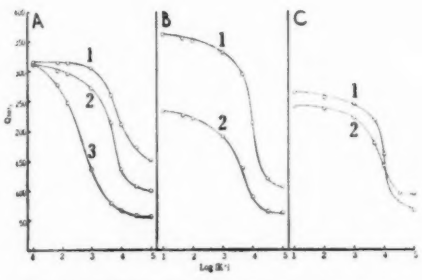

Fig. 4. Effect of K^+ concentration on deamination.

Aspartate and buffers as for Fig. 3.

A. Anaerobic, phosphate + 10^{-3} M glucose; 1, pH 7·35; 2, pH 7·0; 3, pH 6·13.

B. Aerobic, phosphate; 1, pH 7·95; 2, pH 6·13.

C. Aerobic, borate; 1, pH 8·05; 2, pH 6·51.

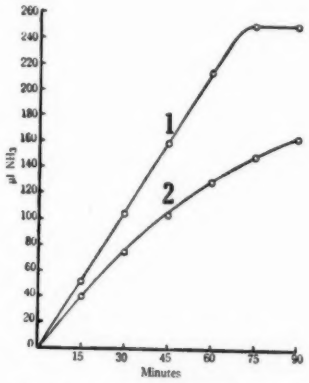

Fig. 5. Course of deamination in low and high sodium media.

250μl aspartate; pH 7·2; Anaerobic.

Curve 1. K:K phosphate + 0·02 M NaCl.

Curve 2. Na:Na phosphate + 0·02 M KCl.

The effect of metal ions on the metabolism of fumarate.

Aspartase in toluene-treated cells or extracts was unaffected by the absence of sodium or potassium ions, suggesting that these ions act indirectly by affecting some metabolic process associated with the integrity of the cell wall. Therefore their effect on fumarate metabolism was investigated (Table 5). Both fermentation and oxidation of fumarate were greatly reduced in the absence of either K^+ or Na^+. However, in contrast to aspartate deamination, fumarate metabolism was not inhibited by high concentrations of sodium.

TABLE 5.

The effect of K^+ and Na^+ on fumarate metabolism.

(0·066 M phosphate, pH 7·2; 0·02 M fumarate; incubation, 60 mins.)

Medium	Changes in μl. Anaerobic Fumarate	Anaerobic CO_2	Anaerobic Succinate	Aerobic O_2
Na:Na phosphate	—260	+117	+120	—95
Na:Na phosphate + 0·02 M KCl	—990	+510	+510	—910
K:K phosphate	—200	+120	+110	—117
K:K phosphate + 0·02 M NaCl	—1030	+530	+560	—907

DISCUSSION.

The effects on aspartic acid deamination of sodium, potassium and hydrogen ions, reported in this paper, are related to the integrity of the cell wall. When the membrane is partly disorganized by treatment with toluene, or freezing and thawing (Trudinger, 1953) or when cell-extracts are used (unpublished results), the requirement for K^+ and Na^+, and inhibition by high concentrations of Na^+, are no longer found. Further, the effect of pH becomes independent of the cationic constitution of the medium. A somewhat similar phenomenon has been described by Fleischmann and Schwarz (1937), who found that, while Li^+ and Na^+ accelerate, and K^+ arrests, methylene blue reduction by yeast cells, these ions have no effect on the reduction of the dyestuff by maceration juices.

In view of the well-known activation of fermentation by K^+ (Farmer and Jones, 1942; Muntz, 1947), it may well be that this ion exerts its effect indirectly by affecting fermentative processes related to deamination. Since fumarate metabolism is dependent on K^+ it suggests that the rate of basic anaerobic deamination may be governed by the rate of fumarate fermentation. The action of Na^+ is probably similar, since fumarate metabolism requires this ion also.

It has been suggested already (Trudinger, 1951) that stimulation by glucose, etc. is related to its fermentation. The metabolism of glucose and adenosine by *Proteus* is dependent on K^+ but not on Na^+ (unpublished results), which may be the reason why *stimulation* of deamination is increased by K^+ in sodium buffer but not by Na^+ in potassium buffer.

One effect of glucose and other stimulators is to offset the inhibitory effect of high concentrations of sodium ions. This may be related to the intracellular concentration of K^+ by fermentation with a concurrent exclusion of Na^+. Such a phenomenon has been observed with red cells (Danowski, 1941; Pulver and Verzár, 1941), yeast (Pulver and Verzár, 1940; Conway and O'Malley, 1946; Rothstein and Enns, 1946; Scott *et al.*, 1951), brain cells (Dixon, 1949; Davies and Krebs, 1952), and bacteria (Lieberwitz and Kupermintz, 1942; Cowie *et al.*, 1949; Roberts *et al.*, 1949).

The mechanism of sodium inhibition of the basic deamination is unknown. Competitive antagonism of K^+ by sodium has been observed in the growth of lactic bacteria (MacLeod and Snell, 1948), but in the case of aspartate deamination, inhibition is independent of the K^+ concentration. Alternatively, it is unlikely that sodium inhibits by preventing K^+ accumulation by mass action since (a) high concentrations of K^+ do not antagonize Na^+ although the latter is equally essential for the deamination, and (b) above a certain level, increases in Na^+ concentration produce no further inhibition of the deamination. Although K^+ and Na^+ may stimulate deamination through their action on fumarate metabolism, inhibition by high concentrations of Na^+ cannot be thus explained, since fumarate metabolism is not affected.

The increased metal requirement with decreasing pH is probably due to cationic exchange between K^+ or Na^+ and hydrogen ions (*cf.* Conway and O'Malley, 1946; Rothstein and Enns, 1946; Conway and Brady, 1950; Robertson, 1950; Maeson and Lako, 1952). The phenomenon may be viewed in two ways. Increases in metal ions may exclude hydrogen ions from the internal environment, thus buffering the internal medium at a more favourable pH for deamination; or an increase in H^+ concentration may expel intracellular K^+ and Na^+ (observed by Eddy and Hinshelwood, 1950), necessitating higher external concentrations of metallic ions to produce the internal levels required for optimal metabolic activity. The absence of inhibition by sodium at low pH values may mean exclusion of this ion from the internal medium by hydrogen ions.

Since either sodium or potassium is already present in the external medium in high concentration as a constituent of the buffer, the latter postulate is considered most likely. Nevertheless, the tendency for the pH curves to "level out" as the metal concentration is increased indicates that they may control to some extent the internal pH. Such a suggestion has been made by Eddy and Hinshelwood (1951), who demonstrated that, at adverse pH values, high concentration of K^+ stimulated glucose oxidation in *E. coli*, but not at physiological pH values. However, deamination of asparagine and alanine did not respond to K^+ at pH values lower than optimal.

SUMMARY.

Intact *Proteus* X-19 requires sodium and potassium ions for maximal deamination of aspartic acid. At pH 7, the optimal concentration for both ions is *ca.* 0·01 M.

K^+ concentrations above 0·01 M increase slightly the rate of deamination, but Na^+ inhibits the basic anaerobic activity above 0·05 M.

Aerobic and stimulated anaerobic deamination are unaffected by high Na^+ concentrations.

The relation between pH and deamination is markedly affected by the ionic constitution of the medium.

Higher concentrations of metallic ions are required for optimal activation at acid pH values than at more alkaline reactions. Small concentrations of K^+ and Na^+ which activate strongly at pH 7 and over may have no effect at pH 6.

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PLASMA AND BLOOD VOLUMES OF NORMAL AUSTRALIAN FEMALES

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(Accepted for publication 12th May, 1953.)

This paper reports values for plasma and blood volumes of normal Australian females obtained with a T-1824 method over approximately the same period as a previously published male series (Steinbeck, 1950).

METHODS.

The subjects were apparently normal medical students and hospital outpatients.

T-1824 samples were those used before and the general and experimental procedures were not altered.

Blood volume was calculated by proportion from plasma volume and an empirically corrected haematocrit (observed venous haematocrit/1·04).

Protein concentration of plasma was estimated after micro-Kjeldahl digestion and direct Nesslerization of digests by standard methods.

RESULTS.

Disappearance of dye from circulating plasma. Semilogarithmic plotting of plasma dye concentrations against the time since injection smoothed disappearance curves for the dye. The concentration of dye on the disappearance line at 30 minutes after injection, expressed as a percentage of the initial concentration derived by extrapolation, indicated the amount of dye remaining in the plasma. Such an expression seems preferable to one of dye loss and the mean value was 95·1 ± 0·46 p.c. (s.e.m.) which was significantly greater ($p<0·01$) than that for males, a difference noted by von Porat (1951). On a weight basis, 0·317 ± 0·011 (s.e.m.) mg./kg. T-1824 were injected in females and 0·285 ± 0·005 in males.

Estimation of plasma volume. Individual estimations are listed in Table 1. The correlation between plasma volume and body weight was significant ($p<0·01$) and the corresponding linear regression coefficient was significantly different from zero ($p<0·01$): variation in plasma volume due to body weight was greater than that within individuals grouped by weight ($p<0·01$). Likewise plasma volume regressed significantly in a linear manner upon body height ($p<0·01$), body surface area ($0·05>p>0·01$) and body weight and height ($p<0·01$). Where $\hat{Y}$ = predicted plasma volume in litres, X_1 = body weight (kg.), X_2 = body height (cm.), X_3 = body surface area (Du Bois, m.^{2}), the

corresponding regression equations and mean squares of random deviations from regression are:

(1) $\hat{Y} = 2{\cdot}742 + 0{\cdot}03639\ (X_1 - 57{\cdot}14)$, $s_{y.x_1}^2 = 0{\cdot}07752$.

(2) $\hat{Y} = 2{\cdot}742 + 0{\cdot}04354\ (X_2 - 163{\cdot}9)$, $s_{y.x_2}^2 = 0{\cdot}08980$.

(3) $\hat{Y} = 2{\cdot}742 + 2{\cdot}596\ \ (X_3 - 1{\cdot}615)$, $s_{y.x_3}^2 = 0{\cdot}06042$.

(4) $\hat{Y} = 2{\cdot}742 + 0{\cdot}02664\ (X_1 - 57{\cdot}14) + 0{\cdot}02759\ (X_2 - 163{\cdot}9)$, $s_{y{\cdot}12}^2 = 0{\cdot}05829$.

TABLE 1.

Plasma volumes of normal females derived from a full dye disappearance curve, 30-minute disappearance curve and 10-minute dye concentrations shown alongside corresponding body variates.

Subject	Age (yrs.)	Height (cm.)	Weight (kg.)	Plasma volume (l.) derived from full dye disappearance curve (a)	30-minute disappearance curve (b)	10-minute dye concentration (c)	Corrected haematocrit (p.c.)
1	22	171·5	64·5	3·24	3·20	3·30	36·7
2	21	171·5	65·0	3·37	3·35	3·39	36·0
3	35	164·9	45·3	2·45	2·41	2·52	43·8
4	32	170·2	70·0	3·09	3·05	3·13	40·1
5	19	170·2	69·5	3·86	3·73	3·79	38·9
6	19	159·6	54·5	2·51	2·51	2·53	40·5
7	21	170·1	62·5	2·94	2·94	2·96	36·5
8	20	169·5	56·0	2·83	2·81	2·87	38·3
9	38	170·2	57·0	3·07	3·08	3·07	38·2
10	21	165·7	65·7	3·00	2·91	2·99	35·5
11	16	158·6	54·0	2·08	2·06	2·12	42·6
12	44	162·0	73·0	3·03	3·02	3·10	42·1
13	15	154·8	54·0	2·55	2·55	2·60	35·6
14	60	154·9	60·0	2·50	2·37	2·34	41·3
15	43	166·0	64·5	3·20	3·20	3·25	38·4
16	23	157·2	51·0	2·46	2·42	2·51	38·2
17	22	157·0	48·3	2·60	2·61	2·67	37·0
18	22	162·7	55·5	2·98	2·92	2·95	38·7
19	21	162·0	53·0	2·96	2·88	2·95	37·5
20	23	156·5	49·6	2·23	2·21	2·29	39·2
21	19	169·5	58·0	2·50	2·49	2·55	40·1
22	19	169·5	54·0	2·87	2·87	2·93	37·4
23	19	165·0	49·5	2·44	2·44	2·47	38·5
24	22	162·7	52·1	2·98	2·92	2·99	37·5
25	20	163·5	56·0	2·86	2·79	2·84	34·5
26	21	169·6	65·9	2·95	2·95	3·00	40·9
27	22	163·5	62·0	2·84	2·84	2·90	37·8
28	24	159·7	53·5	2·36	2·32	2·39	42·5
29	30	174·0	60·5	2·62	2·62	2·70	43·7
30	20	157·3	49·0	2·21	2·21	2·25	38·5
31	22	172·6	59·0	2·92	2·90	2·96	37·3
32	35	165·0	45·4	2·55	2·41	2·50	43·7
33	32	154·9	44·5	2·16	2·32	2·40	38·5
34	15	159·6	47·5	2·16	2·16	2·20	43·1
35	45	157·0	69·5	2·62	2·66	2·72	42·0
Mean	—	164	57·1	2·742	2·718	2·755	39·2
s.e.m.	—	±1	±1·3	±0·066	±0·063	±0·087	±0·43

In addition, plasma volumes were calculated by two other ways—with an initial dye concentration derived from dye concentrations in the first 30 minutes after injection and with the actual 10 minute concentration. These calculations are also shown in Table 1, columns (b) and (c) respectively, alongside the original estimates in column (a). Comparing the values as series, those obtained using a 30-minute disappearance of dye did not differ significantly from those when the longer sampling time was employed and the variances were similar. Volumes calculated from the 10-minute dye level were not significantly different from those obtained with a disappearance curve although the variance was significantly greater ($0{\cdot}05>p>0{\cdot}01$). This suggests that estimations of plasma volume from a single dye level may not be as consistent as by an extrapolation method, and sampling for 30 minutes appears adequate in this respect.

Relative plasma volume. Mean values were $48{\cdot}23 \pm 0{\cdot}87$ (s.e.m.) ml./kg. body weight, $16{\cdot}70 \pm 0{\cdot}34$ ml./cm. height and $1{\cdot}687 \pm 0{\cdot}0265$ l./m.^{2} surface area.

Estimation of blood volume. Blood volume also regressed significantly in a linear manner upon body weight ($p<0{\cdot}01$), height ($p<0{\cdot}01$) and body weight and height ($p<0{\cdot}01$). The corresponding regression equations and mean squares of random deviations from regression where $\bar{U}$ = predicted blood volume in litres are:

(5) $\bar{U} = 4{\cdot}517 + 0{\cdot}05698\ (X_1 - 57{\cdot}14)$, $s_{z.x_1}^2 = 0{\cdot}1603$.

(6) $\bar{U} = 4{\cdot}517 + 0{\cdot}06731\ (X_2 - 163{\cdot}9)$, $s_{z.x_2}^2 = 0{\cdot}1950$.

(7) $\bar{U} = 4{\cdot}517 + 0{\cdot}04215\ (X_1 - 57{\cdot}14) + 0{\cdot}04198\ (X_2 - 163{\cdot}9)$, $s_{z.12}^2 = 0{\cdot}1152$.

Reproducibility of plasma volume. In six subjects, estimation of plasma volume was repeated after six months. The same volume of 0·5 p.c. T-1824 was injected on both occasions and from the same syringe. First, plasma volume was calculated from the 10-minute dye concentration and second from the disappearance curve of dye. These results are shown on Table 2 and it is seen that paired estimates correspond closely. If differences between pairs are considered as deviations from a theoretical zero, the standard deviation is $\pm 0{\cdot}13$ l. for the single sample group and $\pm 0{\cdot}15$ l. for the extrapolation group.

TABLE 2.

Estimations of plasma volume repeated after six months on the same subject. Volumes were derived from (a) 10-minute dye concentrations and (b) full disappearance curves.

Subject (kg.)	Plasma volume (l.) (a) Original	(a) Repeated	(b) Original	(b) Repeated
49·6	2·67	2·51	2·60	2·46
54·3	2·95	2·95	2·96	2·98
64·8	3·39	3·30	3·37	3·24
67·3	4·08	4·04	3·94	4·02
79·3	3·92	3·79	3·87	3·71
89·3	4·12	3·95	4·02	3·91

Plasma protein concentration. The mean total protein concentration was 6·43 ± 0·42 (s.e.m.) gm./100 ml. plasma and the range was 5·86–7·24 gm./100 ml. Total circulating protein correlated significantly with body weight ($p<0·01$) but did not regress significantly.

DISCUSSION.

Extrapolation of dye concentrations to zero time on a semilogarithmic plot corrects for dye-albumin loss (Barnes, Loutit and Reeve, 1948) and lymphatic return of dye-albumin does not introduce a significant error (Courtice, Simmonds and Steinbeck, 1951). The percentage of dye remaining in the circulating plasma at 30 minutes after injection would indicate a loss of some 5 p.c. of dye which is higher than reported mean losses (Gibson and Evans, 1937a; Noble and Gregersen, 1946; Courtice and Gunton, 1949) but similar to that found by Chaplin and Mollison (1952), who used a similar dye sample.

The plasma volumes repeated after six months agreed closely with the originals. This might suggest that either the method is insensitive or that dye-distribution is physiologically constant for normals in the post-absorptive state. The significant difference between repeated values would be 0·4 l. ($p = 0·05$) and this is similar to published maximal variation of repeated determinations (Gibson and Evelyn, 1938; Clark *et al.*, 1947).

TABLE 3.

Comparison of relative plasma volumes, ml./kg. body weight, for normal females.

Author	Method of calculation	Number of subjects	Mean plasma volume (ml./kg.)	Mean square f volumes	Significance of difference between present series
Freis and Kenny (1947)	Semilog plot	6	44·8	—	—
Gibson and Evans (1937b)*	Linear plot	39	42·15	34·70	$p<0·01$
Hallock (1940)	Linear plot	10	39·0	—	—
Harington, Pochin and Squire (1940)	Mean dye concentration	4	44·8	10·15	—
Steinbeck*	Semilog plot	35	48·23	26·44	—
Stewart and Rourke (1944)*	Linear plot	18	44·36	26·12	$p<0·05$
von Porat (1951)*	Linear plot	20	44·7	21·44	$p<0·05$
	15-min. dye concentration	20	44·9	—	—

* No significant difference between male and female series of authors.

Comparable figures have been reported by Gibson and Evans (1937b), Stewart and Rourke (1941) and von Porat (1951). The latter two series represent significantly smaller values than the present ($p<0{\cdot}05$) and the regression statistics are similar. The first series probably also represents smaller values but in this instance variation in plasma volume due to body weight was no greater than that within individuals grouped by weight. If available results are compared as relative volumes, ml./kg. body weight, as in Table 3, it is seen that the present mean value is in excess of most others, as the corresponding male value was. If male and female volumes/kg. body weight are compared there is no significant sex difference with one exception (Hallock, 1940).

SUMMARY.

Plasma volumes have been determined in healthy women by a T-1824 method and blood volumes were derived by proportion with the haematocrits.

The relationship of plasma volume to body variates has been considered and to corresponding regression equations calculated.

A significant difference between repeated estimates of plasma volume is at least 0·4 l.

A comparison with other reported values has been made.

Acknowledgment is made to Professor C. G. Lambie for his surveillance and continued interest.

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OBSERVATIONS ON CARBOHYDRATE METABOLISM AND ALLOXAN DIABETES IN A MARSUPIAL (*TRICHOSURUS VULPECULA*)

by D. MONK ADAMS[1] AND ADOLPH BOLLIGER

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Bolliger and Whitten (1938) noticed that the urine of the common brush-tail phalanger (*Trichosurus vulpecula*) usually contains reducing substances and gives a positive Bial reaction for pentose. These observations suggested an unusual carbohydrate metabolism in this marsupial and further studies of the urine and blood sugar of normal phalangers were, therefore, undertaken. In addition, alloxan was injected into a number of phalangers and the response to this diabetogenic compound compared with that obtained in higher mammals (Eutheria).

METHODS.

Blood was obtained from the marginal ear veins of fasting and non-fasting animals. After the injection of alloxan by the intravenous or intracardiac route blood specimens were obtained usually at 15, 30, 45, 60 or at 15, 37½ and 60 minute intervals. Following these samples, hourly specimens were taken up to eight hours. The course of the ensuing diabetes was followed by daily and later by weekly readings of the blood sugar level. The sugar content of the blood was determined by the method of Somogyi (1945).

Urine can easily be obtained by pressure on the bladder through the abdominal wall, but when handled the phalanger frequently urinates and spontaneously voided specimens can be collected. For the detection of reducing substances Benedict's solution was employed and the urines were tested for pentose by Tauber's reaction (1937). The method of McRary and Slattery (1945) was used for quantitative determination of substances reacting with orcinol usually assumed to be pentoses.

A pure preparation of alloxan monohydrate (Kodak) was made up as an approximately 8–10 p.c. solution in saline at 37° C.

RESULTS.

Normal Phalangers.

Urine. From normal, non-fasting phalangers, urine nearly always contained reducing substances which, expressed as glucose, ranged from a trace to 2·2 p.c. in amount (Table 1). "Pentoses" determined by McRary and Slat-

[1] Aided by a grant from the National Health and Medical Research Council. Deceased 26th April, 1953, aged 33.

tery's method ranged from 0·05 to 1·2 p.c. These colorimetric "pentose" readings, however, were frequently unsatisfactory owing to the formation of a precipitate. Moreover, Tauber's qualitative test was always negative even though the established pentose content of the urine was 0·5 p.c. or more. So far, it has not been possible to demonstrate the presence of pentoses in urine by paper chromatography.

TABLE 1.

Reducing substances expressed as glucose and substances reacting with orcinol expressed as pentose in the urine of the common phalanger (T. vulpecula) *and the effect of hydrolysis on these constituents.*

Phalanger No.	Reducing substances		Substances reacting with orcinol	
	before hydrolysis p.c.	after hydrolysis p.c.	before hydrolysis p.c.	after hydrolysis p.c.
829	0·18	0·40	0·18	
735	0·80	1·00	0·22	
735	0·16	0·35	0·24	0·26
827	0·83	1·04	0·30	
835	0·33	0·59	0·29	0·29
836	0·16	0·44	0·30	0·32
1129	2·20	3·90	0·36	0·76
817	0·07	0·10	0·27	0·23
817	0·06	0·11	0·07	0·08
831	0·37	0·84	0·34	0·40
743	0·70	2·40	1·20	1·37
494	0·20	0·24	0·16	0·25
1028	0·31	0·46	0·23	0·23
351	0·08	0·12	0·06	0·06
Averages	0·47	0·86	0·30	0·36

On hydrolysing urine from normal phalangers by adding 0·2 ml. hydrochloric acid to a specimen of 5 ml. and warming for 2 hours at water bath temperature, reducing substances increased by 83 p.c. on an average, while the alleged "pentose" content did not increase at all or only to a comparatively small extent (Table 1)

On fasting for 24 hours, the reducing substances in the urine disappeared as indicated by Benedict's test. Strangely, however, "pentoses" (0·1–0·3 p.c.) were still present in amounts exceeding the total reducing substances determined by Somogyi's (1926) method.

Blood. In estimations on 17 non-fasting animals the blood sugar ranged from 45 to 126 mg. p.c., averaging 88 mg. p.c. In 12 fasting animals the blood sugar ranged from 60 to 115 mg. p.c., averaging 81 mg. p.c. Hydrolysis did not increase the reducing substances of the blood.

Owing to the small amounts of urine excreted and the difficulties of handling the phalangers, it has not yet been possible to determine accurately the blood sugar value at which reducing substances appear in the urine (renal threshold) but on a number of occasions reducing substances were found to be excreted in

the urine at a glucose blood level of 115 mg. p.c. or less. This would indicate a low renal threshold.

A blood sugar level of over 120 mg. p.c. persisting for at least two days was taken as the criterion of established diabetes.

Phalangers injected with alloxan.

Intraperitoneal injections. Alloxan injected intraperitoneally in doses of 300 mg./kg. into two phalangers or even in doses of 400 mg./kg. into a further two phalangers failed to raise the blood sugar except for a few hours immediately after the administration of the drug. An intraperitoneal dose of 800 mg./kg. proved fatal within six hours of administration.

TABLE 2.

Intracardiac injection of alloxan.

Phalanger No.	Dosage mg./kg.	Duration of Diabetes (days)	Survival (days)	Remarks
1126	200	16	Still living	Recovered from diabetes
735	250	22	Still living	Recovered from diabetes
830	250	0	—	
739	260	12	12	Died in a coma
827	270	0	—	
740	275	20	20	Hypoglycaemic death
724	300	0	—	
487	300	14	14	Died in a coma
828	300	15	15	
376	300	2	2	Acute alloxan intoxication
832	330	2	2	

Intracardiac injections. Eleven animals, most of them under ether anaesthesia, were injected intracardially because at the time it was believed that the intravenous route would be impracticable. As shown in Table 2, eight of these phalangers receiving from 220 to 330 mg./kg. of alloxan developed diabetes according to our criterion. Two of these animals (376 and 832) died 48 hours after the injection of an obviously excessive dose of alloxan. Phalangers 739, 487 and 828 died in coma within two weeks. Another phalanger (740), whose blood sugar rose to 500 mg. p.c. a week after the injection of alloxan, died on the twentieth day of the experiment in severe hypoglycaemia (blood sugar level 21 m.g. p.c.) inadvertently brought on by an overdose of insulin. The autopsy of these six animals revealed extensive damage to kidneys and liver and widespread fatty degeneration in addition to the varying degrees of necrosis of the β-cells in the islet of Langerhans.

Phalangers 735 and 1126 recovered from the mild diabetes sustained from an intracardiac alloxan injection three weeks previously and are still alive. Their highest observed blood sugar readings were 265 and 186 mg. p.c. respectively.

Intravenous injections. To produce diabetes by this route the minimum effective dose is about 115 mg./kg. of alloxan (Table 3). A dose as high as 175

mg./kg. still seemed to be safe, since in the present series no acute deaths occurred attributable to this amount of alloxan injected. Insulin hypoglycaemia and an accidental haemorrhage were responsible for the death of phalanger 833, which lived for 12 days only after an injection of 115 mg./kg. of alloxan. Four of the six animals in this group developed diabetes. Phalanger 803, which was not affected by a dose of 100 mg./kg. given eight weeks earlier, responded to 150 mg./kg. Phalanger 1126 had recovered from a mild diabetes brought on by 220 mg./kg. alloxan given intracardially three months earlier. Now 115 mg./kg. given intravenously brought on a persistent diabetes. With dosages from 200 to 300 mg./kg., five out of six phalangers died within two weeks and all these showed severe renal and some liver damage. Phalanger 741, which survived the immediate effects of alloxan, developed subsequently a very severe diabetes with blood sugar levels above 400 mg. p.c. which could be controlled for a period of three months by a daily injection of 4 units of zinc protamin insulin.

TABLE 3.

Intravenous injection of alloxan.

Phalanger No.	Dosage mg./kg.	Fasted	Duration of diabetes (days)	Survival (days)	Remarks
743	50	—	0	—	
817	100	+	0	—	
803	100	+	0	—	
833	115	+	12	12	Accidental death
1126	115	+	140	Still living	
803	150	+	124	Still living	
817	150	+	0	—	
745	165	—	115	Still living	
834	175	—	0	—	
732	200	—	10	10	
819	200	—	20	20	
802	265	+	6	6	Died in coma
741	275	+	90	90	
742	275	+	0	—	
388	325	+	1	1	Acute alloxan intoxication

Of the 16 phalangers which became diabetic after intracardiac and intravenous injections only two after intracardiac administration (735 and 1126) recovered completely from the disease. All the others which survived the first fortnight after alloxan administration began to lose weight. Some required insulin while others managed for 15 to 30 weeks without medication. Injections of insulin, however, ultimately became necessary in every instance to protect the animal from wasting and coma.

Response of blood sugar level immediately after alloxan injections. The level of blood sugar immediately after alloxan administration varied greatly in

different phalangers (Figs. 1, 2 and 3). The responses on the day of alloxan injection in those 12 animals where this period was studied fall into two groups.

In the first group, represented by one quarter of these experiments, alloxan produced no diabetes subsequently, with the exception of phalanger 803, which had two injections separated by three months, an ineffective one of 100 mg./kg. and an effective one of 150 mg./kg. In the animals of this group the blood sugar showed only slight changes (Fig. 1), never rising above 120 mg. p.c. during the 7 hours following alloxan injection.

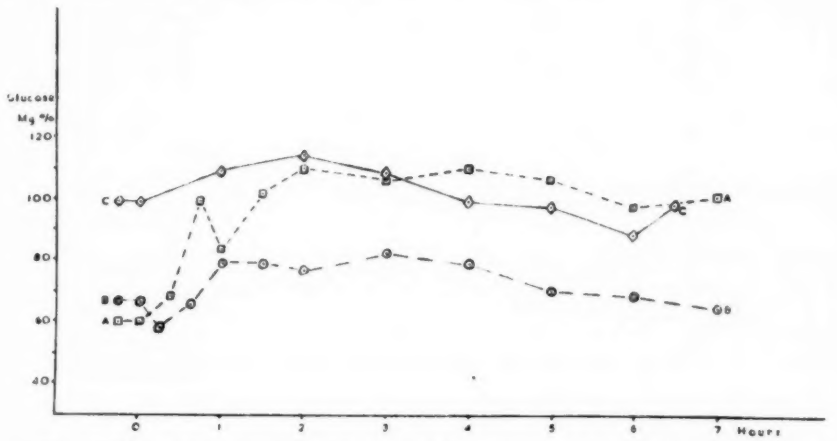

Fig. 1. Blood sugar curves of phalangers which showed no definite immediate response after alloxan administration, inasmuch as the blood sugar remained within normal limits for seven hours.

A = experiment 803 (150 mg./kg. intravenously).
B = experiment 803 (100 mg./kg. intravenously).
C = experiment 817 (150 mg./kg. intravenously).

Only in A did the animal subsequently become diabetic.

In the second group hyperglycaemia occurred in four animals within the first 30 minutes after injection. In two further animals, where the first determination after alloxan was not made until after the lapse of two hours, the blood sugar was well above 200 mg. p.c. and it is conjectured from the curves of Fig. 2 that no hypoglycaemic phase had occurred. The elevated blood sugar in these six phalangers persisted indefinitely. It thus appeared that diabetes was always produced when this early rise of the glucose content of the blood was observed. The ensuing diabetes was of varying degrees of severity and in some instances (experiment 741) insulin had to be given even a few days after the injection of alloxan in order to save the animal from coma. Diabetic phalangers respond readily to insulin, and severe hypoglycaemia after its administration must be guarded against.

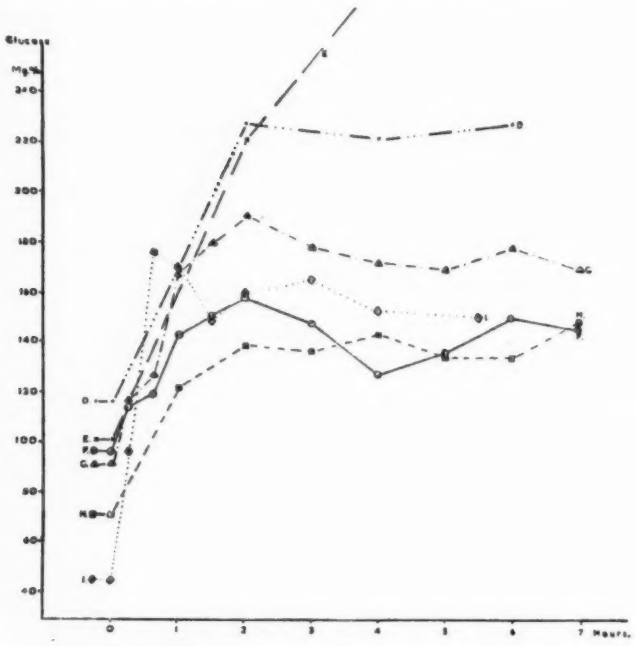

Fig. 2. Blood sugar curves of phalangers which showed an immediate hyperglycaemic reaction after the administration of alloxan.

D = experiment 832 (330 mg./kg. intracardiac).
E = experiment 739 (260 mg./kg. intracardiac).
F = experiment 745 (165 mg./kg. intravenously).
G = experiment 732 (200 mg./kg. intravenously).
H = experiment 833 (115 mg./kg. intravenously).
I = experiment 376 (300 mg./kg. intracardiac).

In all these experiments permanent diabetes developed.

Fig. 3 depicts atypical immediate blood sugar responses of three phalangers. All these three animals became diabetic. Two were distinguished by the fact that after an initial rise the blood sugar fell below the pre-experimental levels in the third or fourth hour after alloxan administration. This fall in blood sugar reached a definite hypoglycaemic level in phalanger 1126 (Fig. 3), which several months previously had an intracardiac injection of alloxan which did produce a mild, short-lived diabetes. It is noteworthy that this experiment is the only one in our series in which a few hours after alloxan administration a true though very transient hypoglycaemia was observed. In the third of these three phalangers (experiment 487) the blood sugar level fell immediately after the intracardiac injection of 300 mg./kg. and remained subnormal for a period of two hours.

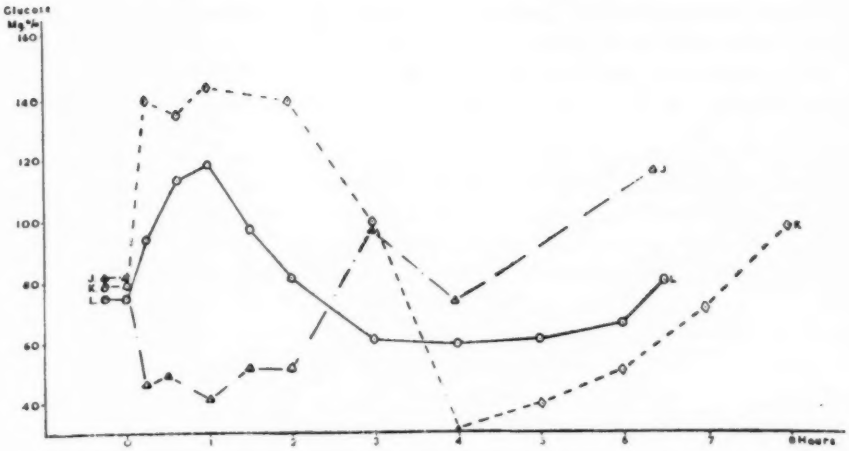

Fig. 3. Shortlived hypoglycaemic reaction in three phalangers in the first, third and fourth hours respectively after alloxan administration. These reactions do not correspond with the usual more severe hypoglycaemic phases observed in Eutheria.

J = experiment 487 (300 mg./kg. intracardiac).
K = experiment 1124 (115 mg./kg. intravenously).
L = experiment 819 (200 mg./kg. intravenously).

All three animals became subsequently diabetic.

TABLE 4.

Reducing substances expressed as glucose and substances reacting with orcinol expressed as pentose in the urine of the diabetic phalanger, T. vulpecula, *and the effect of hydrolysis on these constituents. The analysed urine specimens were obtained on varying diets at approximately weekly intervals.*

Phalanger No.	Reducing substances		Substances reacting with orcinol	
	before hydrolysis p.c.	after hydrolysis p.c.	before hydrolysis p.c.	after hydrolysis p.c.
739	1·04	3·59		
1126	1·54	2·67	0·59	0·53
,,	0·59	1·06	0·55	0·51
,,	1·78	4·07	0·26	0·32
803	4·20	7·40	0·40	0·40
,,	1·72	2·74	0·18	0·35
,,	3·32	6·55	0·41	0·44
,,	4·92	7·85	—	—
745	3·23	5·76	0·60	0·54
,,	2·20	2·56	—	—
Averages	2·51	4·43	0·43	0·44

The urine of alloxan diabetic phalangers. Reducing substances were always present even in the fasting animal, and expressed as glucose were found to reach levels as high as 12 p.c. The unusual feature, however, was that on hydrolysis

with hydrochloric acid at water bath temperature the reducing substances always increased considerably (Table 4). The "pentose" content ranged from 0·2 p.c. to 0·7 p.c. and increased only slightly on hydrolysis. The result of the Tauber reaction for "pentose" was doubtful, the colour being a strong dark brown instead of red.

Animals with blood sugars over 200 mg. p.c. frequently yielded a strongly positive Rothera test for ketone bodies. In most of these instances a positive ferric chloride test was also obtained indicating the presence of considerable amounts of aceto acetic acid. Hydrolysis did not increase the amount of reducing substances present in the blood of the diabetic animal.

DISCUSSION.

The fasting blood sugar of the common phalanger lies within the range found in other mammals, but the renal threshold is low and reducing substances are nearly always present in the urine of the non-fasted animals. The bulk of these reducing substances is not pentose, as originally suggested (Anderson, 1937) because they do not give a positive Tauber reaction and pentoses could not be demonstrated with paper chromatography. A quantitative adaptation of Bial's test (McRary and Slattery, 1948) indicated that a fraction of the reducing substances could be pentose, but this seems to be an artifact because in the urine of the fasting animal the values for the alleged pentose greatly exceeded the total reducing substances. Therefore it seems that the substance in the urine reacting with orcinol and ferric chloride (Bial's test) is in all probability not pentose or any other reducing substance.

In the diabetic animal with pathological glycosuria Tauber's test for pentose is doubtful and similar to that seen in human diabetics without pentosuria. In addition, pentose values as indicated by the colorimetric method employed here are not higher than in the normal animal where the Tauber reaction for pentose was negative.

On hydrolysis the amount of reducing substances in the urine of normal and diabetic animals increases considerably (Tables 1 and 4). This would indicate that a large part of the reducing substances is combined and becomes liberated on hydrolysis. According to paper chromatography the reducing substance obtained by hydrolysis, like that initially present, seems to be glucose.

These observations indicate the unusual nature of the reducing substances in the urine of the normal and the diabetic *Trichosurus vulpecula*. Somewhat similar conditions were encountered in the milk of the kangaroo (Bolliger and Pascoe, 1953).

As in other mammals the intravenous route is the method of choice for the administration of alloxan (Lukens, 1948), and when the seemingly very small ear veins of phalangers were found to be suitable for this purpose the intracardiac administration was abandoned.

Intravenous administration of a dose from 115–175 mg./kg. was found to be non-lethal but diabetes ensued in only four out of the six phalangers. With larger doses from 200 to 275 mg./kg. only one animal which survived the early acute period, characterized by severe renal insufficiency combined with diabetes, could be kept alive for three months with the help of insulin.

In many respects the effects of alloxan injections in phalangers show considerable agreement with those observed in eutherian mammals. On the other hand the progressive nature of the diabetes produced in these marsupials is on the whole greater than that produced with similar procedures in most higher mammals. Only two phalangers recovered from alloxan diabetes in contrast to rabbits where recovery is more frequent.

The blood sugar response immediately after alloxan administration, however, is definitely different from that observed in the Eutheria. Typically the phalangers exhibited two main types of response. In the first type the blood sugar remained essentially within normal limits for some eight hours after alloxan administration and diabetes ensued only once—in an animal which had a second alloxan injection.

In the second type the blood sugar immediately after alloxan administration rose to diabetic levels and this elevated blood sugar persisted or became even more pronounced within the period of observation, which extended for as long as 40 weeks in some animals.

These effects are in marked contrast to the so-called triphasic blood sugar curve response after the intravenous administration of diabetogenic doses of alloxan in higher mammals. This "eutherian" curve consists firstly of a rise in blood sugar at 2 to 4 hours after the injection. This is followed by a marked and frequently lethal hypoglycaemia at 5 to 12 hours and a permanent hyperglycaemia at 18 to 24 hours. This pattern of response has been observed by many workers on at least 7 eutherian species. The hypoglycaemia and the subsequent diabetic phase could always be noticed in such higher mammals while the initial hyperglycaemia may not always occur. The hypoglycaemia is usually so severe as to necessitate the administration of glucose to prevent death a few hours after the administration of alloxan (Lukens, 1949).

All our animals survived the first 24 hours of the experiment without administration of glucose and in only one instance was a transiently dangerous low blood sugar encountered near the critical period (experiment 1126). In another experiment, a moderate hypoglycaemia was noted during the first hour but not in the 5–12 hour period (experiment 487, intracardiac injection). This is reminiscent of the hypoglycaemia observed in dogs during the first 45 minutes after alloxan (Shipley and Beyer, 1947).

In all other instances either an essentially flat (Fig. 1) or a steeply rising and persistently diabetic curve was observed in the phalanger (Fig. 2).

It remained obscure why at a certain dose of alloxan some of these animals became diabetic and others not; why some responded with a blood sugar curve

of type 1, some with type 2. These effects could not be explained by sex or by the nutritional status of the experimental animals because whether they were fasted or not fasted and whether lean or fat, did not seem to influence the result.

Similar problems have not been solved in the order Eutheria and the causation of the triphasic sugar response after alloxan administration is still the subject of considerable controversy. The ensuing severe hypoglycaemia has been explained by the most generally accepted hypothesis—that preformed insulin is liberated from the damaged islet tissue. However, Adams (1953) has shown that the hypoglycaemic response may be abolished in the rabbit with normal liver glycogen content if the pancreatic ducts are ligated several weeks before the alloxan administration. Thus a somewhat similar response is obtained with both the phalanger, a marsupial, and a higher mammal with its pancreatic duct ligated. Whether this is a clue to the peculiarities of carbohydrate metabolism observed in a marsupial remains to be seen.

Owing to the untimely death of Dr. D. Monk Adams certain questions arising from this investigation are as yet unanswered. It is hoped to resume the work, incorporating some of the suggestions which the late Dr. Adams had made.

SUMMARY.

The urine of the common brush-tail phalanger, *Trichosurus vulpecula*, a herbivorous diprotodont marsupial, nearly always contains reducing substances which normally disappear on fasting and which seem to consist mostly of glucose. The presence of pentoses could not be established.

The amount of reducing substances in the urine of normal and diabetic phalangers increases markedly on hydrolysis, apparently due to combined glucose.

Alloxan was administered intraperitoneally, intracardially and intravenously to phalangers. After the intracardiac and intravenous administration diabetes resulted in nearly three-quarters of the animals receiving suitable doses. An intravenous dose of 115–175 mg./kg. was found to be most satisfactory.

With the exception of two animals which had intracardiac injections the resulting diabetes was persistent and frequently insulin had to be injected to avoid wasting and coma.

The immediate response of the blood sugar level of the phalanger to diabetogenic doses of alloxan is different from that observed in other mammals. A typical hypoglycaemic phase was not observed and in most instances diabetes became established immediately. In those cases where diabetes did not follow alloxan administration the immediate response curve was within normal blood sugar levels.

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A PROTHROMBIN-FIBRINOGEN COMPLEX AND ITS RELATION TO PROTHROMBIN ESTIMATIONS

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The methods for estimating prothrombin concentration in human plasma have been a controversial subject for many years because theoretical opinions and practical observations cannot be reconciled.

It is believed that the experimental data described herein offer an explanation of and correlate:

(a) the varying results in prothrombin estimation in hypoprothrombinaemia;

(b) the close agreement of the results obtained using either the two-stage method or the one-stage Russell viper venom method in prothrombin estimation;

(c) Quick's observation that normal plasma diluted with prothrombin-free plasma maintains a constant prothrombin time of 12 seconds until the prothrombin concentration has been reduced by more than half;

(d) the participation of both free and inert prothrombin in the clotting process as described by Quick (1949);

(e) the decrease in prothrombin time which follows the clotting of haemophilic blood;

(f) some results concerning factor VI and serum prothrombin conversion accelerators (Owren, 1947; Alexander *et al.*, 1951);

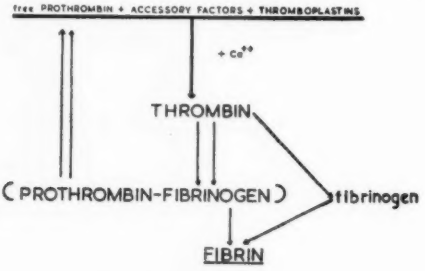
(g) severe haemorrhage due to dicoumarol therapy when relatively large amounts of prothrombin can be isolated from the blood.

The subsequent experiments are described to substantiate the existence of a prothrombin-fibrinogen complex (Lyons, 1952) and to provide evidence to suggest that prothrombin and fibrinogen exist in *human* blood both as a complex and in the free state. It appears that the prothrombin of the complex is inert until the fibrinogen moiety has been acted upon by thrombin. The amount of prothrombin which is then released from the complex is almost twice as great

[1] Work done under a grant from the National Health and Medical Research Council.

as the original concentration of free prothrombin. Hence the potential prothrombin concentration of plasma is well over 200 p.c.

The hypothesis is best described by the following diagram:



Reagents and Methods.

Brain thromboplastin. The method of Quick (1951) was used for both human and rabbit brain. These preparations were used only when they gave a prothrombin time of 11–12 seconds for normal plasma.

Russell viper venom activated brain. One half gm. of dry powdered brain prepared by the method of Quick was mixed with a solution of 0·1 mg. of Russell viper venom in 2 ml. of physiological saline. After incubation for 20 minutes at 37° C. 30 ml. of acetone were added. The suspension was filtered using a small Buchner funnel, dried by suction and evaporation and then 100 ml. of acetone were poured over the dry powder. After slow filtration the powder was again dried by sucking air through the filter.

The dry powder was then suspended in 10 ml. of physiological saline and heated for 20 minutes at 50° C. After cooling to 37° C. this suspension was ready for use and was found to be of undiminished activity after storage for 7 days at —30° C.

Normal human plasma gives a prothrombin time between 7 and 8 seconds when this thromboplastin is used. This activity is not due to any retained Russell viper venom.

Other reagents. Russell viper venom–lecithin was prepared according to the method of Mawson (1949).

The term "oxalated phosphate plasma" is used to emphasize that oxalate ions are in excess in the final mixture of plasmas or sera so that the concentration of Ca^{++} is no more than that of calcium oxalate.

Other reagents used in these experiments were prepared as previously described (Lyons, 1951).

Apart from the few experiments necessary for comparative purposes, all blood and thromboplastins used were of human origin.

RESULTS.

The Method of Quick (1935).

This method has given rise to a great deal of controversy over many years, but is now generally accepted as the most useful for clinical work. It is based upon the assumption that the prothrombin concentration is related to the clotting time of recalcified plasma when thromboplastin is added in excess.

In spite of this assumption a peculiar anomaly is observed if we apply another test suggested by Quick (1949), the prothrombin consumption test, to the serum derived from his one-stage method: the prothrombin concentration apparently increases instead of becoming negligible. The prothrombin indices obtained then agree with those obtained by the Russell viper venom + lecithin method and by the two-stage method. The theoretical and experimental values are in close agreement also.

When the prothrombin time is estimated by Quick's method, it will be found that when the first clot is removed immediately and the serum is expressed, a second clot forms in 4–5 seconds in this serum. If this clot is removed, no further clotting is observed. This phenomenon may be due to a free and combined form of fibrinogen.

The possibility that the low prothrombin time of 6–8 seconds is due to thrombin formed during the first one-stage estimation is not likely in view of the fact that the addition of *oxalated* calcium phosphate plasma (pH 7·3) results in a clotting time of 15 seconds. However, when a mixture of equal parts of calcium phosphate plasma, brain thromboplastin and calcium chloride is added immediately the second clot is removed, a prothrombin time of less than 8 seconds is observed (Table 1).

TABLE 1.

Splitting of the prothrombin-fibrinogen complex.

	Normal Plasma (ml.)	Prothrombin-free plasma (ml.)	Haemophilic serum (fresh)* (ml.)	Trypsin 1:100,000 (ml.)	Brain thromboplastin (ml.)	Russell viper venom 0.1 mg/ml. (ml.)	Incubation period† (secs.)	Prothrombin time (secs.)
(1)	0·1	—	—	—	0·1	—	—	12
(2)	0·05	0·05	—	—	0·1	—	—	12
(3)	0·05	0·05	—	—	0·05	0·05	20	7–8
(4)	0·05	—	—	0·05	0·1	—	20	7–8
(5)	—	0·05	0·05	—	0·1	—	—	8
(6)	0·05	—	—	0·05	0·05	0·05	20	7–8
(7)	Fresh serum from (1) 0·05‡	0·05	—	—	0·1	—	Nil	6–8

All plasma mixtures above were recalcified by adding 0·1 ml. of M/40 calcium chloride.

* Obtained less than one hour after the clotting of haemophilic blood.

† Incubation period is only quoted where any change has been noticed in the prothrombin time after incubation of mixtures which lacked one essential ingredient for clotting.

‡ Within 5 secs. of clotting, clot removed and serum obtained.

Experiments performed have consistently indicated (1) that the prothrombin concentration increased after clotting, and (2) that desiccated brain and Russell viper venom used as thromboplastic agents did not act in the same manner. The difference in the mode of action was even more apparent when both were used together. The remarkable prothrombin time of four seconds was

obtained when normal oxalated plasma was incubated at 37° C. for twenty seconds with a mixture of both these thromboplastic agents and then recalcified (Table 2).

TABLE 2.

A minimum prothrombin time.

Normal oxalated plasma (ml.)	Russell viper venom (0·1 mg./ml.) (ml.)	Brain thrombo-plastin (ml.)	Incubation period at 37° C.* (secs.)	Clotting time (secs.)
0·5	0·25	0·25	20	4
0·5	0·5	—	20	21
0·5	—	0·5	20	12
0·5	0·25	0·25	—	12

0·05 ml. of M/40 $CaCl_2$ was added to each mixture.

* This is the minimum incubation period. No difference was observed when an incubation period of 300 seconds was used.

Whilst the results typified by Table 2 indicate a different mode of thromboplastic action for brain and venom it is apparent that the action was not a simple one. Viper venom requires some time between 12 and 20 seconds to exert its effect. It was possible for the venom to have acted upon the brain "thromboplastin," upon the plasma, or upon both during the 12–20 seconds' incubulation period required.

Further investigation suggested that the Russell viper venom acts upon both the brain "thromboplastin" and the oxalated plasma. Desiccated brain tissue, prepared by Quick's method, could be altered by a brief incubation with a solution of Russell viper venom. The treated brain tissue freed from venom by washing with acetone produced a prothrombin time of eight seconds compared with twelve seconds when untreated *provided that it was incubated with the oxalated plasma for twenty seconds* before recalcification.

When venom-treated brain was incubated for longer than twenty seconds the prothrombin time did not decrease. This was interpreted to mean that the effect was due to the altered brain tissue and not to lingering traces of venom. It had been established that a combination of brain and venom would lower the prothrombin time of normal plasma to four seconds under these conditions. The cumulative and separate effects of Russell viper venom on both oxalated plasma and brain may be seen in Table 3.

It would appear that if the accelerated clotting time which is observed when Russell viper venom and brain thromboplastin are used together were due to a prothrombin conversion accelerator, there would be a great difference in clotting time when the resultant serum was added together with thromboplastin and calcium ions, to prothrombin-free plasma and to normal plasma. No difference was observed even subsequently when the serum from these mixtures was added in reverse order.

TABLE 3.

Normal oxalated human plasma (ml.)	Brain prepared according to Quick (ml.)	Brain treated with Russell viper venom (ml.)	Russell viper venom (0·1 mg./ml.) (ml.)	Incubation period at 37° C.* (secs.)	Prothrombin time (secs.)
0·5	0·5	—	—	Nil	12
0·5	0·5	—	—	20	12
0·5	—	0·5	—	Nil	12
0·5	—	0·5	—	20	8
0·5	—	0·5	—	300	8
0·5	0·25	—	0·25	Nil	12
0·5	0·25	—	0·25	20	4
0·5	—	0·25	0·25	20	4
0·5	—	0·25	0·25	Nil	8
0·5	0·25	—	0·25	300	14
0·5	—	0·25	0·25	300	16
0·5	—	—	0·5	20	21
0·5	—	—	—	—	190

* 0·5 ml. M/40 $CaCl_2$ was added to each mixture after the prescribed incubation period.

The absence of a prothrombin-fibrinogen complex in afibrinogenaemia.

A patient with afibrinogenaemia has been available for study for one year and it has been impossible to demonstrate any evidence of a prothrombin-fibrinogen complex. When afibrinogenaemic blood was mixed with fresh prothrombin-free plasma no shortening of the prothrombin time was observed.

In addition the free prothrombin of his blood was apparently not greatly affected as a prothrombin time (equal volume of prothrombin-free plasma added) of 12–13 seconds was frequently recorded. No evidence of fibrinolysis was present.

The results of the various prothrombin estimations on this patient's blood are set out in Table 4.

These findings indicate that there is a normal concentration of free prothrombin in this afibrinogenaemic blood, yet there is no reserve of prothrombin combined with fibrinogen such as in normal blood.

TABLE 4.

Prothrombin estimation in afibrinogenaemia.

No.	Afibrinogenaemic plasma (ml.)	Normal plasma (ml.)	Prothrombin-free plasma (ml.)	Russell viper venom (0·1 mg./ml.) (ml.)	Brain thromboplastin (ml.)	Activated brain thromboplastin (ml.)	Incubation period at 37° C. (secs.)	Clotting time (secs.)
1	—	0·1	—	—	0·1	—	—	12
2	—	0·1	—	—	—	0·1	20	8
3	—	0·1	—	0·05	0·05	—	20	4
4	0·1	—	—	0·05	0·05	—	20	No clot
5	0·05	0·05	—	0·05	0·05	—	20	7
6	0·05	—	0·05	—	0·1	—	—	12
7	0·05	—	0·05	0·05	0·05	—	20	12
8	0·05	—	0·05	0·05	0·05	—	20	11·5

The action of Russell viper venom in conjunction with brain as a thromboplastic agent.

The anomalies noted in the introductory paragraphs are not as striking as the observation made by Quick and others that when normal human plasma is diluted with prothrombin-free plasma the prothrombin time does not increase, until the prothrombin concentration is decreased to under 50 p.c. Quick attributes this to a factor present in calcium phosphate plasma which regulates the balance between free prothrombin and prothrombinogen, keeping the concentration of free prothrombin at 100 p.c. until the reserve of prothrombinogen is depleted (Quick, 1951).

It is difficult to understand why this adjusting factor should be present only in prothrombin-free plasma and not in the plasma containing both free and bound prothrombin. It does not necessarily follow that both brain and venom have the same mode of action. By mixing both venom and brain and using this mixture as a thromboplastin it was discovered that even half usual quantities would give a prothrombin time of four seconds—one third that of the usual one-stage method (see Table 5).

TABLE 5.

Comparison of thromboplastins over prothrombin range 100–10 p.c.

Thromboplastic agent												
	Normal plasma, ml.	0·1	0·09	0·08	0·07	0·06	0·05	0·04	0·03	0·02	0·01	0·00
	Prothrombin-free plasma, ml.	—	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10
Rabbit brain*	Clotting time, secs.	12	12	12	12	12	12	13	14	19	26	—
Human brain	Clotting time, secs.	10	10	10	10	10	11	13	15	19	30	—
Activated human brain	Clotting time, secs.	8	8·5	9	9·5	10	10·5	12	14·5	19	26	—
Human brain + Russell viper venom	Clotting time, secs.	4	4	4	5	5·5	7	8·5	10·5	14	26	—

* Quoted by Quick (1951).

Many workers have had difficulty in producing standardized brain thromboplastin. It is suggested that this may be overcome by treating the dried brain with Russell viper venom and then repeating the acetone treatment (see preparation of reagents). The resultant brain powder, whether of human or rabbit origin, yields a thromboplastin which is much more potent than that prepared by any other method, and which permits the accurate estimation of the decrease in prothrombin when normal plasma is mixed with prothrombin-free plasma *in vitro* over the whole range of dilution. As a result of these observations it is suggested that if desiccated brain tissue is treated with a solution of Russell

viper venom a thromboplastin is produced (in contact with plasma) which can measure the prothrombin concentration in the 100–50 p.c. range. Fig. 1 compares the method of Quick (which shows no variation in prothrombin time in the 100–50 p.c. range) with the new method which is sensitive over the whole range. The thromboplastin suspension used in compiling the graph cited as Quick's method in Fig. 1 was made from rabbit brain according to Quick and gave similar results to those quoted by Quick (1951).

DISCUSSION.

A one-stage method of prothrombin estimation, which has been found more sensitive than Quick's method for determining free prothrombin, may throw light upon the reactions involved in "thromboplastic" activity.

The results recorded in this paper indicate that both rabbit and human brain contain an active principle in a relatively inert form which can be activated by Russell viper venom in the absence of Ca^{++}. This activated brain tissue then needs at least a 20-second incubation period with oxalated plasma before the actual thromboplastin is formed. Subsequent investigation of the oxalated plasma and the suspended brain tissue indicates that the *substance now possessing the thromboplastic power is the plasma* and that the brain, in excess as usual, still has the power to activate more oxalated plasma but has not acquired any new powers whereas the plasma has.

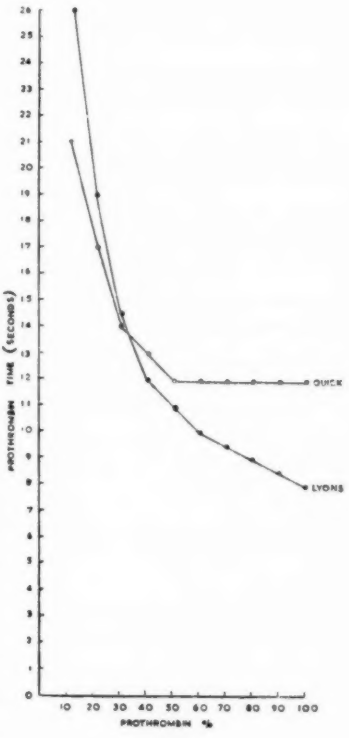


Fig. 7.

The estimation of prothrombin concentration, according to Quick, shows no difference between normal plasma and normal plasma plus an equal volume of prothrombin-free plasma. Hence it appears that Quick's method is insensitive to changes in prothrombin concentration in the range of 100–50 p.c. Our experimental results indicate that this insensitivity can be overcome when the same brain "thromboplastin" is treated with Russell viper venom. Experiments which have been carried out during the last two years in the investigation of a possible prothrombin-fibrinogen complex have always confirmed and never denied its existence. The action of Russell viper venom upon brain "thromboplastin" was a coincidental finding

in an investigation of this prothrombin-fibrinogen complex. As a result of these findings it is suggested that Russell viper venom has more than one mode of action in the estimation of the prothrombin time. A study of the results obtained using this venom either separately or in conjunction with brain "thromboplastin" have suggested that it acts upon an inert prothrombin liberating more free prothrombin and also that it acts upon the brain thromboplastin itself.

The use of Russell viper venom alone as a thromboplastic agent gives disappointing results because the prothrombin times are inconsistent with, more prolonged than, and have a less definite end point than those obtained by Quick's method.

The use of venom plus lecithin as suggested by Hobson and Witts (1941) gives a prothrombin time which varies between 6·5 and 8 seconds for normal plasma.

This prothrombin time is not the minimum, however. In this laboratory we have consistently noted prothrombin times of less than 4·5 seconds for normals when the thromboplastic agent was a mixture of equal parts of Russell viper venom and human brain thromboplastin.

Whenever Russell viper venom is used in conjunction with any other agent, plasma should be incubated with it for 15–20 seconds to obtain a minimal prothrombin time. When brain and venom are used together as thromboplastic agents, the resultant prothrombin time of normal human plasma varies with the order of mixing and the incubation period.

Results obtained using the two-stage method for prothrombin estimation and the Russell viper venom plus lecithin method agree closely.

This study, which is only of a preliminary nature, has also shown that the free prothrombin (estimated by Quick's method) is rapidly affected by adequate doses of dicoumarol, and that the prothrombin in the suggested complex is not affected for about 48 hours. The prothrombin of this complex appears to be estimated together with the free prothrombin when either the venom-lecithin or the two-stage method is employed.

Free prothrombin appears to be reduced very rapidly by dicoumarol therapy. When the free prothrombin *concentration* falls below 10 p.c., the thrombin, which is formed slowly, is even more quickly inactivated by the plasma antithrombins. Yet under these circumstances prothrombin can still be isolated from the blood and it is suggested that this prothrombin was part of a prothrombin-fibrinogen complex before attempts were made to isolate it.

SUMMARY.

It is suggested that prothrombin and fibrinogen occur in normal blood both as a complex and as separate entities, and that prothrombin of the complex can be liberated either by clotting, by the action of Russell viper venom, or by trypsin.

Experimental data appear to provide an explanation of the discrepancies between the one- and two-stage methods of prothrombin estimation.

A one-stage method of prothrombin determination is described which is believed to be more sensitive than Quick's method over the range of 100–50 p.c.

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THE LIMITED GROWTH OF INFLUENZA VIRUSES IN THE MOUSE BRAIN

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Schlesinger (1950) has shown that inoculation of large quantities of unadapted influenza virus into the mouse brain results in the production of non-infective haemagglutinin ("incomplete" virus) without any comparable production of "complete" infective particles. He took this behaviour of the virus as the basis for its unadaptedness and consequent failure to grow logarithmically. However, small inocula have since been shown to undergo one cycle of multiplication with production of "complete" virus (Cairns, 1951). Superficially therefore the behaviour of unadapted strains in mouse brains resembles, at least for the first cycle, the behaviour of egg-adapted virus in the allantois where small inocula yield "complete" virus only, and large inocula mainly "incomplete" (von Magnus, 1946; Cairns and Edney, 1952).

Further investigation, reported in this paper, has shown that small inocula probably produce no "incomplete" virus in the first cycle, and certainly do not render the brain incapable of supporting the multiplication of a second inoculum. From this it follows that the failure of unadapted strains to grow for more than one cycle cannot be attributed to interference by an excess of "incomplete" virus produced in the first cycle.

Materials and Methods.

Virus strains. WS (Smith, Andrewes and Laidlaw, 1933); PR8 (Francis, 1934). Both these strains have been through repeated passage in the allantois and both have at some time been adapted to kill mice on intranasal inoculation. Both show limited multiplication after intracerebral inoculation of adult mice (Cairns, 1951).

Brain suspensions. Each mouse brain was suspended in 1·5 ml. physiological saline, after grinding with alundum.

Titration of infectivity. Serial 3·16-fold (log 0·5) dilutions of the test material were inoculated into groups of three 10–12 day eggs. After 3 days' incubation at 35° C., each allantoic fluid was tested for the presence of haemagglutinin. Titres were expressed as allantoic-ID_{50} per brain.

Titration of haemagglutinin in brain suspensions. For this it was necessary both to destroy the haemagglutinin-inhibitor which is normally present in brain suspensions (Schlesinger, 1949) and to employ as sensitive a technique as possible by using the minimum amount

of red cells and the maximum amount of test material. Each brain suspension was treated therefore with 150 units of receptor-destroying enzyme (Burnet and Stone, 1947) for 2 hours at 37° C. and then lightly centrifuged. Serial 0·5 ml. doubling dilutions of the supernate were made in citrate saline to stop the action of the receptor-destroying enzyme (Fazekas de St.Groth, 1948), and for the same reason a period of cooling in the refrigerator was allowed. Then 0·04 ml. 1 p.c. fowl red cells was added to each tube and, after 2 more hours in the refrigerator, the pattern of settled cells was read. This technique is twelve times as sensitive as the standard test in which equal volumes (0·25 ml.) of the test dilutions and red cells are brought together.

EXPERIMENTAL.

Production of "complete" and "incomplete" virus by small and large inocula.

Although Schlesinger's contention, that unadapted strains of influenza virus will produce no viable offspring in the mouse brain, was not borne out by the experiment (Cairns, 1951), it still seemed possible that further multiplication of the infected particles produced in the first cycle might be interfered with by an excess of "incomplete" virus released at the same time. Unfortunately, owing to the low sensitivity of even the improved haemagglutinin test, it is impossible to compare directly the yields of "complete" and "incomplete" virus particles (that is of infectivity and haemagglutinin) after small inocula which are known to generate at least some infective virus. However, as all workers in the field agree that the large inoculum differs from the small one only in the relative portions of "complete" and "incomplete" virus produced, it should be possible to infer how much of the latter is being liberated by studying the yields relative to inoculum size. After large inocula the haemagglutinin test will measure the sum of infective and "incomplete" virus;

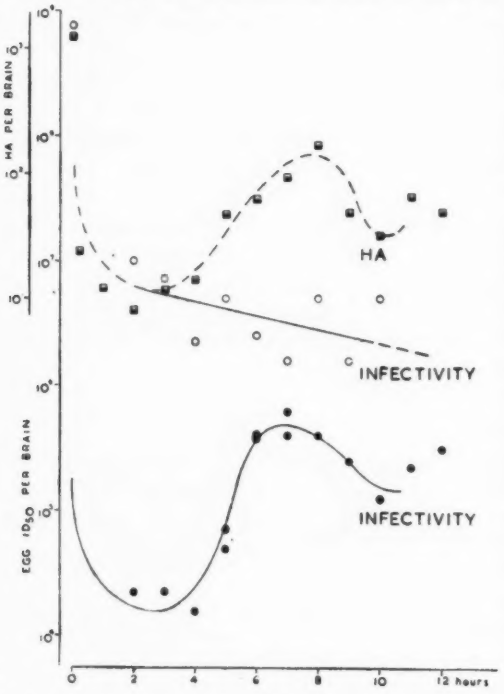

Fig. 1. Multiplication of PR8 virus in mouse brain. Production of haemagglutinin and infective virus following a large inoculum and of infective virus following a small inoculum.

after small inocula the number of "complete" virus particles can be assessed by infectivity. If the relative yields (in proportion to inoculum size) of infective virus after small inocula and total haemagglutinin after large inocula are the same—and this proves to be the case—there will be no need to postulate the production of any "incomplete" virus by the small inocula.

A group of four-weeks-old mice was inoculated with 0·03 ml. undiluted PR8 virus. Two mice were killed after ten minutes and from then on two mice were killed every hour. The brain suspensions from each pair of mice were pooled and titrated for haemagglutinin and infectivity. In Fig. 1 the results are compared with the changes in infectivity found after inoculating virus diluted $10^{-2\cdot5}$ (Cairns, 1951).

In confirmation of Schlesinger's findings (1950), no detectable rise in infectivity titre is seen after the larger inoculum, showing that this inoculum evoked the production of "incomplete" rather than "complete" virus. Secondly, it is apparent that during the first four hours the resting levels of unadsorbed non-multiplying virus are, like the original inocula, separated by about 2·5 log units, showing the percentual uptake of virus in a given time is independent of the amount of virus inoculated; a similar finding has already been reported for the allantois (Henle, 1949). After the large initial inoculum the haemagglutinin titre rose $10^{1\cdot2}$-fold by the seventh hour above the resting level; the corresponding rise in infectivity after the smaller infective dose was $10^{1\cdot6}$-fold. Thus, relative to the inoculum size and the resting level, the large inoculum is clearly producing less "incomplete" virus than the small is producing "complete"; for the reasons outlined above, this suggests that the small inoculum is not producing any "incomplete" virus. It would appear then that, as for the allantois (Cairns and Edney, 1952), large inocula mainly produce "incomplete" virus while small inocula only produce "complete" virus. The slight deficit in haemagglutinin yield by the large inoculum is probably due to saturation of the susceptible cells; investigation of the relation between inoculum size and resulting seventh-hour yield of haemagglutinin has shown that the relative yield is just starting to decline when inocula of this size are used. (Cairns, unpublished.)

Inasmuch as the evidence provided by this experiment is of a negative nature, it does not formally dispose of the possibility that unadapted strains undergo auto-interference in their second cycle by an excess of "incomplete" virus produced in the first cycle; however, such an explanation for their limited growth seems to be ruled out by the experiment reported in the next section.

The multiplication of successive inocula.

One possible cause for the limited growth of unadapted strains would be the production, during the first cycle, of some inhibitor of multiplication, whether this is interfering "incomplete" virus or not. This possibility was readily tested by investigating the capacity of the brain to support successively the

limited growth of two inocula, the second being given at a time when the first had run its full course of multiplication. For this experiment the strain WS was chosen because of all strains tested (Cairns, 1951) it shows the greatest drop in titre after the single cycle and therefore would allow the clearest demonstration of any subsequent cycle of multiplication due to a second inoculum.

A number of four-weeks-old mice was inoculated intracerebrally with approximately 10^5 allantoic-ID_{50} of WS-infected allantoic fluid. Thirteen hours later each mouse received a second, identical inoculum into the same cerebral hemisphere. Groups of four mice were then killed at hourly intervals, and the infectivity of the pooled brain suspensions titrated.

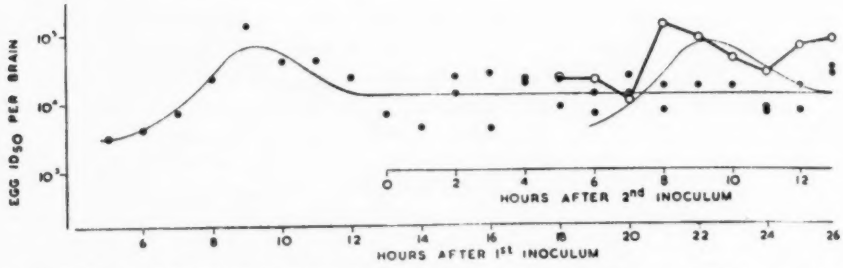

Fig. 2. Multiplication in mouse brain of two inocula of WS virus. The changes in infectivity following a single inoculum (dots) are compared to the changes following a second inoculum (circles) given 13 hours after the first.

Fig. 2 shows the results superimposed on the growth curve for a single inoculum obtained in a separate experiment. It is clear that the second inoculum went through its single cycle of "complete" virus production, even though this was occurring in brains already containing virus at the end of a growth process. It is evident that one inoculum does not, at the same time as it completes its growth process, render the brain refractory to further virus growth.

DISCUSSION.

In a previous paper (Cairns, 1951) unadapted non-neurotropic strains of influenza virus were shown to undergo one cycle of "complete" virus production after intracerebral inoculation. For the first cycle, their behaviour was indistinguishable from that of an adapted neurotropic strain. However, after the first cycle the unadapted strains showed little further multiplication whereas the adapted strain continued to grow logarithmically. Various mechanisms could possibly account for this difference between adapted and unadapted strains.

1. Unadapted strains might produce, in their first cycle, excessive amounts of non-infective virus (or some other growth inhibitor) which would then block

the multiplication of any "complete" virus produced at the same time. This possibility seems to have been excluded by the results reported in this paper. Firstly, small inocula do not produce any "incomplete" virus; secondly, the brain is not refractory to further virus multiplication at a time when one inoculum has completed its limited growth.

2. Unadapted strains might fail to produce some co-factor required for continued fruitful multiplication. It has been suggested that "incomplete" virus production by the allantois is the result of some co-factor deficiency (Cairns and Edney, 1952), and the critical operation of some such factor must be considered here.

3. The virus produced by unadapted strains may fail to multiply in the secondarily infected cells owing to some intrinsic defect of first-generation virus. This possibility demands that the infectivity of virus particles for brain cells may vary without any corresponding variation in their infectivity for the allantois. Such unilateral infectivity has been demonstrated for other viruses (see Bodian, Morgan and Schwerdt, 1950), but it is more usual to find infectivity highest for the homologous tissues rather than the reverse. The striking exception to this rule is the phenomenon recently described by Luria and Human (1952). Coli-phage T2, grown on a particular strain of *B. coli*, will yield a phenotypic variant incapable of continued growth in that host. Yet, the offspring of the single cycle T"2 is genotypically unaltered, i.e. when grown on a different host for which it is fully infective, it will again yield T2 virus. Unfortunately, the analogy could not be submitted to a critical test for technical reasons. The obvious experiment, subinoculation of first-cycle brain virus into fresh brains, is impracticable as one has to start with weak inocula to avoid the presence of "incomplete" virus in the yield, then on subinoculation there is a 95 p.c. loss by leakage (Cairns, 1951), and a further twentyfold reduction of titre due to the fact that one cannot inoculate a whole mouse brain intracerebrally into another mouse.

4. The above possibilities all deal with the situation where the virus enters cells after the first cycle but fails to multiply in these cells. The alternative must be listed that first-cycle virus of unadapted strains fails to enter fresh cells owing to some defect in its power to spread. Thus inoculated virus infects immediately adjacent cells but cannot pass from these cells to others at the end of the first cycle. Although some strains show a sharp drop in titre at the end of the first cycle, suggesting that uptake into fresh cells is occurring, there is no conclusive evidence with which to support or refute this possibility.

At the moment it is impossible to distinguish between the last three alternative explanations for the behaviour of unadapted strains in the mouse brain. However, it is clear that, in this case, the problem of adaptedness is not simply one of sheer capacity to multiply in the particular type of cells.

SUMMARY.

The conditions which lead to the production of "incomplete" virus by unadapted strains of influenza virus in the mouse brain do not appear to be fundamentally different from those obtaining in the allantoic cavity.

The limited growth of unadapted strains in the brain is not associated with the development, by the brain, of refractoriness to further multiplication of a second inoculum.

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OBSERVATION ON THE METABOLISM OF SHORT CHAIN SATURATED FATTY ACIDS BY GUINEA-PIG CAECAL TISSUE

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It has been known for a long time that when fatty acids are incubated with liver or kidney slices acetoacetate is formed. In the last few years ketone body production from fatty acids in the presence of cell-free extracts of these organs has been intensively studied (see Lehninger, 1952). Recently it has been shown that, when short chain saturated fatty acids are incubated with sheep rumen tissue, ketone bodies accumulate in the medium at the expense of the acids (Pennington, 1952). In view of similarities of function of guinea-pig caecum and sheep rumen (Hagen and Robinson, 1953), we thought it would be interesting to observe whether ketone body production took place when guinea-pig caecal tissue was incubated with short chain fatty acids.

This communication reports a study of the ketone body production and oxygen consumption when guinea-pig caecal tissue is incubated with small quantities of the sodium salts of acetic, propionic, butyric, valeric, caproic and heptoic acids.

METHODS.

Guinea-pigs were killed by a blow on the head. Their caeca were immediately removed and slit at either end so that the contents could be washed out with ice-cold 0·9 p.c. (w/v) sodium chloride solution. The caeca were then placed in ice-cold saline solution and cut into several pieces, which were washed and rewashed until the washings were quite clear. Finally, the tissue was chopped into fine pieces with scissors; these were dried between filter paper and 100 mg. placed into Warburg flasks, each containing 3 ml. of Krebs-Ringer phosphate buffer and having KOH and filter paper in the centre well. The fatty acid as the sodium salt at pH 7 was placed in the side arm of the flask. The flasks were shaken in a bath at 37° C. Respiration was usually allowed to proceed for an hour before tipping in the acid. This procedure was adopted in the first experiments when it was thought that a quantitative correlation might be made between oxygen uptake and acetoacetate production and that the initial oxygen consumption without substrate might serve as measure of actively metabolizing tissue in each flask; later the procedure was continued in order that subsequent experiments might be compared with earlier ones. Shaking was continued for a further two hours after adding the substrate and oxygen consumption was measured at 15-minute intervals throughout this period.

After incubation, the flask contents were emptied into centrifuge tubes, each flask washed with 3 ml. water and the washings added to the tubes. After centrifugation, 4 ml. of the supernatant fluid were used for ketone body determination by the method of Greenberg and

Lester (1944), by which acetoacetate and betahydroxybutyrate are estimated as acetone. In each single experiment 100 mg. of the caecal tissue of one animal were put into each of four flasks; one flask served as a control, one contained butyrate in the side arm and each of the other two held another acid as substrate.

In each of two further experiments the caeca of two guinea-pigs, washed as described and cut up together, were used; 750 mg. of the chopped tissue were placed in each of four flasks containing 4 ml. Krebs-Ringer phosphate buffer. The first flask served as a control; to the other three were added 100 micromoles of acetate, propionate and butyrate respectively. These were incubated for two hours and their ketone body content estimated; oxygen uptake was not measured.

RESULTS.

Oxygen consumption and ketone body production during the incubation of 100 mg. of guinea-pig caecal tissue with the various fatty acids are shown in Table 1.

TABLE 1.

Oxygen consumption and ketone body production following incubation of guinea-pig caecal tissue with fatty acids.

Control		Micromoles of substrate added	Acetate		Propionate		Butyrate		Valerate		Caproate		Heptoate	
qO_2*	Ac†		qO_2	Ac	qO_2	Ac	qO_2	Ac	qO_2	Ac	qO_2	Ac	qO_2	Ac
0·68	0·19	50	0·72	0·23	0·67	0·14	0·92	0·76						
0·73	0·11	100	0·84	0·21	0·77	0·01	1·09	0·66						
1·0	0·13	100					1·47	2·10	1·08	0·18	0·99	0·44		
0·79	0·24	50					1·83	2·42	1·09	0·21	1·08	1·70		
0·96	0·32	8					1·39	1·38			1·24	1·03	0·75	0·22
0·91	0·23	8					1·26	0·81					1·15	0·21

* qO_2 represents microlitres of oxygen consumed per mg. (wet weight) of tissue per hour, measured over the two-hour incubation period after adding substrate.

† Ac represents micrograms of acetone produced per mg. (wet weight) of tissue during the three-hour incubation period.

Oxygen uptake was considerably increased in the presence of butyrate and, with lowered substrate concentration, in the presence of caproate. With the other acids used no significant difference from the oxygen uptake of the controls was seen. Ketone body production occurred in all flasks; it was greatly increased in the presence of butyrate and to a lesser degree in the presence of caproate. In one experiment the presence of propionate almost completely abolished the production of ketone bodies. Acetate, valerate and heptoate showed very little difference from the controls. There was no quantitative correlation between oxygen consumption and ketone body production.

Ketone body production after incubating 750 mg. of caecal tissue with acetate, propionate and butyrate is shown in Table 2. Results are given as micrograms of acetone recovered from each flask after two hours' incubation.

TABLE 2.

Ketone body production (as μg. acetone) by guinea-pig caecal tissue (750 mg.) with added substrates (100 micromoles) in 2 hours.

Control	Acetate	Propionate	Butyrate
58	43	16	460
93	85	13	320

From these experiments it appears that acetate has no significant influence on ketone body production by this tissue, while in the presence of butyrate relatively enormous quantities of ketone bodies are produced. With propionate there is a marked reduction in the production of acetoacetate, actually to between a third and a seventh of the control values.

DISCUSSION.

The production of ketone bodies in the absence of added substrate suggests that, like liver, guinea-pig caecal tissue normally contains some precursor of acetoacetate, possibly butyrate absorbed from the lumen of the organ (Hagen and Robinson, 1953) or possibly in the form of other metabolic products in the caecal cells. The high production of ketone bodies in the presence of the even numbered fatty acids, butyrate and caproate, might be expected to take place by reactions similar to those occurring in mammalian liver and kidney (see Green, 1951) or sheep ruminal tissue. The failure to find an exact correlation between oxygen consumption and ketone body production is in line with the findings of previous workers who used liver tissue (Jowett and Quastel, 1935).

An antiketogenic action of propionate in this tissue is a most interesting finding; it has, however, been previously reported in liver slices (Quastel and Wheatley, 1933). Jarrett, Potter and Filsell (1952) showed an antiketogenic effect of propionate in intact sheep. This might well be attributed to the glucogenic activity of this acid in the intact animal such as was demonstrated in rabbits by Ringer (1912) and Pollock (1922), although Pollock claimed that this effect was brought about by an adrenalin-like mobilization of liver glycogen by propionate rather than being a true glucogenesis from propionate. Reid (1950) and Jarrett, Potter and Filsell (1952) have demonstrated a similar glucogenic activity in the sheep. More recently, Daus, Meinke and Calvin (1952) fed isotopically labelled propionate to mice and found a major part of the activity in glucose and the glucogenic compounds, alanine and lactic acid. Huennekens, Mahler and Nordmann (1951) showed that propionate can be converted *via* lactate to pyruvate. These findings, although able to account for the antiketogenic action of propionate in the intact animal and possibly also in liver tissue, do not necessarily account for such antiketogenic activity in isolated guinea-pig caecal tissue. One or two alternative explanations present themselves: either propionate is converted to pyruvate as in liver, in which case its

antiketogenic activity would fall into line with current theories of antiketogenesis (see Baldwin, 1952), or it may be metabolized through some undiscovered pathway, exerting its antiketogenic effect by some other mechanism.

SUMMARY.

Small quantities of ketone bodies accumulate when guinea-pig caecal tissue is incubated in the Krebs-Ringer phosphate buffer in the absence of added substrate.

Ketone body production can be considerably increased by the addition of butyrate and caproate, especially the former, whereas propionate is markedly antiketogenic.

Oxygen consumption is increased in the presence of butyrate and to a lesser degree in the presence of caproate.

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